STUDIES ON PHENOLASE COMPLEX OF *Papaver Somniferum*

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This is to certify that this thesis is the original work of the candidate done under my supervision and is suitable for submission for the award of Ph.D. degree in Biochemistry.

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Dedicated to my mother

SAMIN-E-ZEHRA

who has consoled me in disappointment

and cheered me in success

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ABSTRACT

While trying to isolate the enzymes, responsible for the biosynthesis of opium alkaloids, a distinct phenolase complex system has been recognised in P.somniferum. By the phenolase complex is meant that pair of enzymatic activities occuring together, associated with copper protein and responsible for the o-hydroxylation of phenols and dehydrogenation of o-diphenols.

The phenolase complex of P.somniferum differs from laccase in that it does not catalyse the oxidation of quinol, venillin and p-phenylenediamine. It differs from tyrosinase because it does not oxidise tyrosine either in presence or in absence of added catechol. However, it catalyses the oxidation of DOPAMINE, DOPA, catechol, p-cresol and tyramine. It differs from tyrosinases so far known because it does not oxidise tyrosine either in presence or in absence of added catechol. This distinct phenolase complex is distributed in all the tissues of P.somniferum studied which included roots, leaves, stems, buds and fruits.

The phenolase complex of P.somniferum was inhibited by sodium diethyldithiocarbamate, salicylaldoxime, potassium ethyl xanthate and thiourea which suggested that the enzyme is a copper containing protein. The enzyme was also inhibited by metallic ions which are known to compete with copper such as Ag^{+}, Hg^{++} and Au^{+++}. These results together with other observed evidences suggested that phenolase complex of P.somniferum is a copper containing protein.

In order to gain more insight into the nature of the phenolase complex of P.somniferum, detailed kinetic studies were undertaken.

The formation of quinones during the oxidation of DOPAMINE, DOPA, catechol, p-cresol and tyramine, by phenolase complex of P.somniferum has been demonstrated. The anilino derivatives of quinones, i.e. dianilino-o-benzoquinone and dianilinohomoquinoneanil, produced by the action of phenolase complex on catechol and p-cresol, respectively, have been isolated and identified. The isolation of these derivatives as the products of catechol and p-cresol oxidation in presence of phenolase complex indicated that o-benzoquinones are formed under these conditions and first step

in the oxidation of monophenols is o-hydroxylation.

The oxidation of DOPAMINE, DOPA and tyramine, formation of o-benzoquinones and eventual deposition of black reaction products suggested that the pathway for melanin biosynthesis may be operative in P.somniferum.

The enzyme has been purified 47-fold from the acetone powder extracts of P.somniferum using ammonium sulphate precipitation method, CM-cellulose and DEAE-cellulose chromatography. The properties of partially purified preparations have been studied. With all the phenolic compounds mentioned above, except tyramine, straight lines have been obtained in Lineweaver and Burk reciprocal plot. With tyramine as substrate the reciprocal plot indicated substrate inhibition. The Km values obtained with these preparations show that phenolse complex of P.somniferum has highest affinity for DOPAMINE in comparison to other mono- and di-phenols tested.

The properties of phenolase complex in relation of melanin biosynthesis and the failure to demonstrate the presence of DOPA decarboxylase and amine oxidase in P.somniferum in relation to the biogenesis of opium alkaloids have been discussed.

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DOPA	3,4-dihydroxy-phenylalanine
DOPAMINE	3,4-dihydroxy-phenyl-ethylamine
NADP	Nicotinamide adenine dinucleotide phosphate
CM-cellulose	Carboxymethyl cellulose
DEAE-cellulose	Diethylaminoethyl cellulose
I.R.	Infra red
U.V.	Ultraviolet

IV. INTRODUCTION

Oxidation of Phenols

The enzymic oxidation and coupling of phenols is a subject of considerable importance in biochemistry because biosynthesis of a wide range of natural products including lignins, melanins, antibiotics and alkaloids are known to involve oxidation and coupling of phenols (1,2). The rapid browning of cut or damaged surfaces of fruits and vegetables is caused by the products of enzymic oxidation and coupling of phenolic constituents (3). The enzymic oxidation and polymerization of phenols may also be responsible for the protection of damaged plants from fungal and viral infections. It is believed that the polymers thus formed may inhibit the enzymes of invading microorganisms (4). It is suggested that browning reactions are concerned with wound healing (5,6,7). It has been concluded, from the experiments in which the added substrate was catechol, protocatechuic acid, gallic and caffeic acid, tyrosine, DOPA, phenol, p-cresol, 3,4-dimethyl

catechol (8,9,10,11,12), extracts of potatoes containing naturally occuring phenolic substances (8) or chlorogenic acid (13), that the phenolase complex plays a major role among terminal oxidases in white and sweet potatoes. The role of phenolase complex in terminal oxidation has also been analysed by means of selective inhibitors (14,15,16,17,18).

In order to gain a thorough understanding of the above biological processes it is necessary to know the specificites, physiochemical properties and kinetics of phenol oxidising enzymes. Unfortunately, very little information is available about such enzymes. The most difficult problem presented to a reviewer of the enzymic oxidation of phenols is the classification of the well investigated, but ill understood, enzymes such as laccase (p-diphenol:oxygen Oxidoreductase (EC 1.10.3.2)) (19),peroxidase (donor: hydrogen peroxide oxidoreductase (EC 1.11.1.7.)(20) and tyrosinase (phenolase complex or o-diphenol: oxygen oxidoreductase (EC 1.10.3.1.) (19,20). There is much evidence to favour the sub-division of these three classes of phenol oxidising enzymes since each family differs somewhat in its physical, chemical and biochemical properties (21,22,23,24).

Enzymes Catalysing Oxidation of Phenols - As discussed above the three main classes of enzymes, known to catalyse phenol oxidation, are laccases, peroxidases and phenolase complex. Several species of fungi and bacteria possess these enzymes which are responsible for the production of metabolites which could arise from the oxidation and coupling of phenols. Whether these enzymes belong to the three main classes or whether they have different characteristics is yet unknown. Investigations designed to isolate and study the properties of these enzymes would undoubtedly prove fruitful for the understanding of the coupling reactions that occur in nature.

1. LACCASES

Laccase was first detected in 1883 by Yoshida in the latex of Japanese lacquer tree Rhus vernicifera (25). Its substrate specificity and the nature of the products resulting from simple phenols were investigated by Bertrand (26,27). Subsequently, Keilin and Mann (28) considerably purified the enzyme preparation and established that it was copper containing

protein. More recent investigations are concerned with the physicochemical properties of purified enzyme preparations (29,30,31,32) and structures of the products formed (21,33,34).

(i) Occurrence - The early investigations of Bertrand and later works (21,29,32,35) show that laccases are widely distributed in plant kingdom. They are known to occur in wood rotting fungi and in bacteria (37,38,39,40,41,42). However, the evidence for its occurrence in animal kingdom is sparse and unsatisfactory (43,44,45,46,47,48,49). The methods used to investigate the enzymes are important because the value of claims to have detected and distinguished the enzyme from other phenol oxidases is very much dependent upon the type of investigation used.

(ii) Physico-chemical Properties - Table I summarizes some of the properties of laccases. Laccases consist of proteins coordinated to copper. Laccases from Rhus vernicifera and Polyporus versicolor contain four atoms of copper per molecule and have similar ultraviolet absorption spectra, but different molecular weights and pH optima (21, 29, 30). The laccases from

Rhus vernicifera and Rhus succedanea differ slightly in the position of their long-wavelength ultraviolet absorption bands (32).

(iii) Mechanism - The oxidation of hydroquinone by laccase of R.vernicifera (29)may be represented by the Equation I.

Equation I:

$$2\ Cu^{++}\ (\text{laccase}) + \text{hydroquinone} \longrightarrow 2Cu^{+}(\text{laccase} + 2H^{+} + \text{p-benzoquinone}$$

$$2Cu^{+}\ (\text{laccase}) + \tfrac{1}{2}O_2 + 2H^{+} \longrightarrow 2Cu^{++}(\text{laccase}) + H_2O$$

$$\text{Hydroquinone} + \tfrac{1}{2}O_2 \longrightarrow \text{p-benzoquinone} + H_2O$$

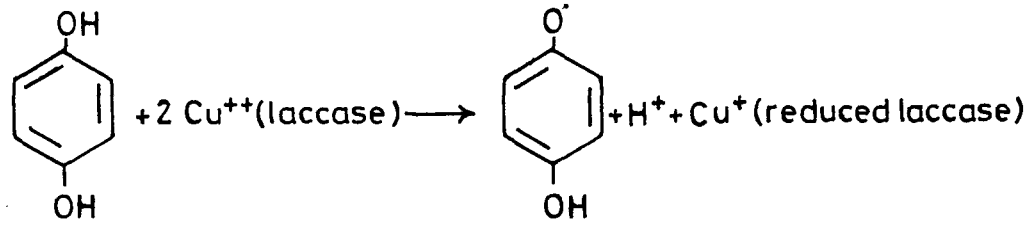
Table I

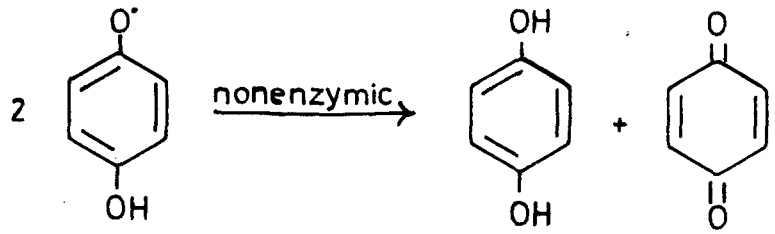
Properties of Laccases

Source	Molecular weight	Cofactor	λmax at pH 7.0	pH optima for 2,6-dimethoxy-phenol
			(m μ)	
Rhus vernicifera	120,000	Cu^{++}	280, 615	6.8
Rhus succedanea	-	-	280, 609	-
Polyporus versicolor	63,000	Cu^{++}	280, 615	4.0

Nakamura (50) has investigated the laccase catalysed oxidation of hydroquinone with the aid of an Electron Spin Resonance spectrometer. He observed that the spectrum is that of the aryloxy radical (I), whose formation is accelerated by laccase (Eq. II). This radical dimutates into hydroquinone and p-benzoquinone, the hydroquinone reacting with more laccase to regenerate the aryloxy radical.

Equation II:

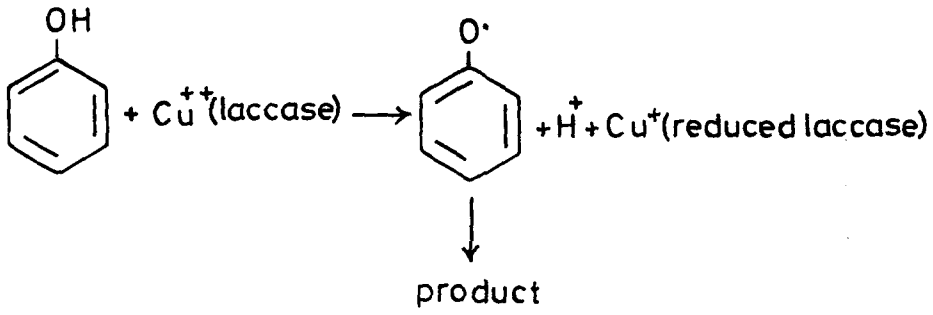




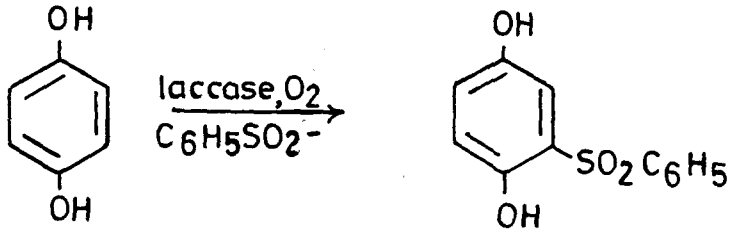
The fact, that laccases act as one electron oxidants and that their action on a variety of phenolic substrates parallels the action of one electron oxidants (21) which are known to produce aryloxy radicals (51,52,53), enables Nakamura's mechanism involving an aryloxy radical to be

applied generally to action of laccases on ıs (Eq.III).

Equation III:

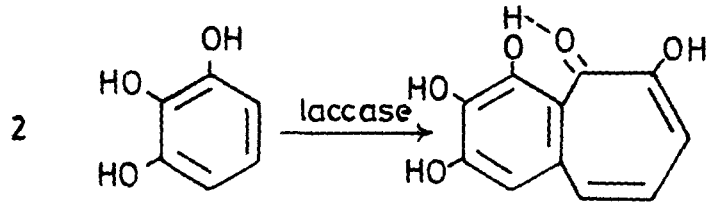


(iv) Products - The only product identified from the laccase catalysed oxidation of hydroquinone is p-benzoquinone (26). Oxidation catalysed by the laccase from Polyporus versicolor and from Rhus vernicifera in the presence of sodium benzenesulfinate yeilds the sulphones (22).



Laccase catalyses the oxidation of catechol and DOPA in the same way as does phenolase complex. It catalyses the oxidation of pyragallol to purpurogallin and this reaction

has been used for the assay of all the three enzymes.



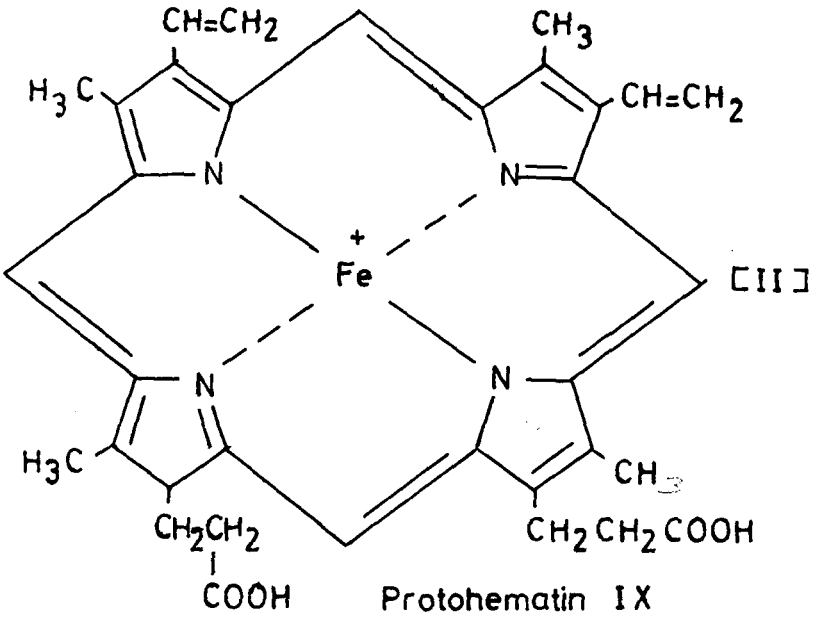
pyrogallol purpurogallin

2. PEROXIDASES

Peroxidases catalyze the oxidation of various substrates, including phenols, by hydrogen peroxide.

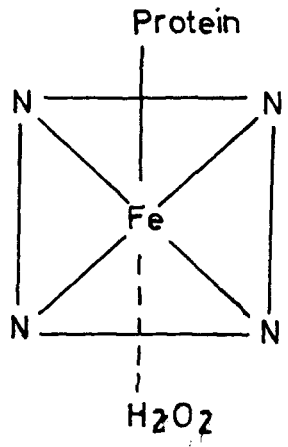
(i) Occurrence - The enzyme has been detected in plants, fungi, bacteria and animals. Several different peroxidases have been isolated and identified, some of them in crystalli state, for example, horseradish peroxidase (54), myeloperoxi from leucocytes (55) and lactoperoxidase from milk (56).

(ii) Physico-chemical Characterstics - Table II gives a summary of the important characteristics of the enzyme. Horse-radish peroxidase consists of a protein (apoenzyme) together with an iron porphyrin compound, protohematin IX (II), as coenzyme (57). The enzyme also contains carbohydrate. The iron atom is thought to be surrounded octahederally by the porphyrin, the protein and another ligand for example H_2O_2 (III) (55,57,58).



Protohematin IX

[II]



[III]

(iii) Mechanism - Electron spin resonance measurements (59) have established that oxidation of hydroquinone by the peroxidase-hydrogen peroxide complex produces the aryloxy

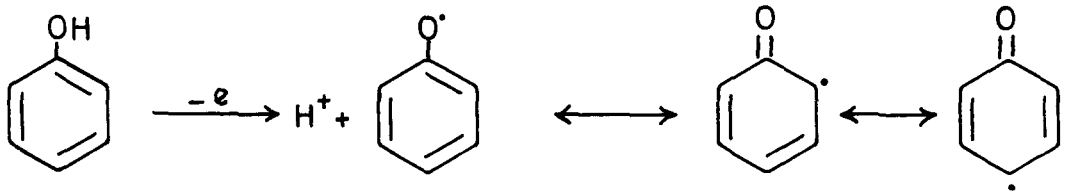
Table II

Properties of peroxidases

Source	Molecular weight	Iron Content	Coenzyme
		(percent)	
Horse radish	40,000	0.127	Protohematin IX
Leucocytes	149,000	0.074	Two iron porphyrin
Milk	93,000	0.07	Unknown porphyrin(s)

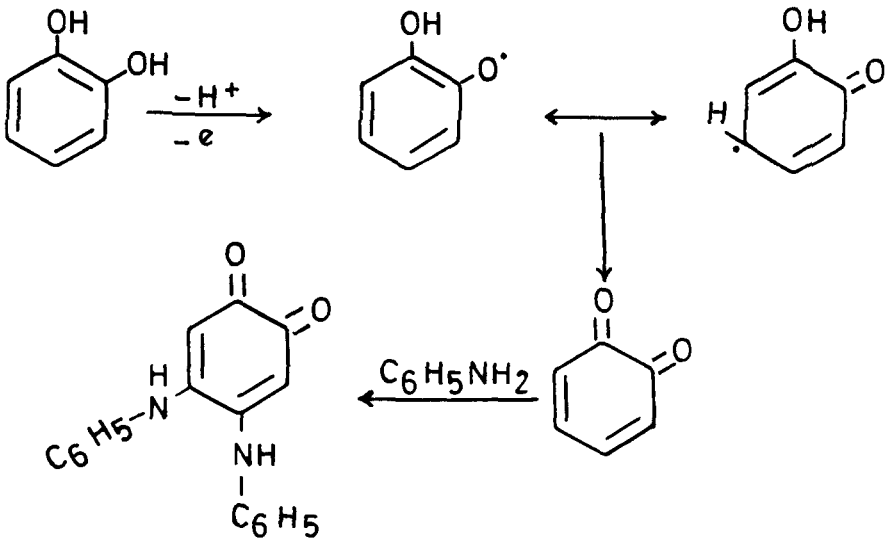
radical. This confirms earlier spectrophotometric (60) and potentiometric (61) studies which established that the peroxidase complex acts by a one electron oxidation mechanism (Eq.IV).

Equation IV:



(iv) Products - The products of peroxidase catalyzed oxidations of phenols, usually parallel those produced chemically by one electron oxidants and therefore aryloxy radicals appear generally to be intermediates.

Pough and Raper (62) isolated the anilino compound (IV) from peroxidase catalyzed oxidation of catechol with H_2O_2



[IV]

Peroxidase catalyses the oxidation of pyragallol to purpurogallin in presence of hydrogen peroxide.

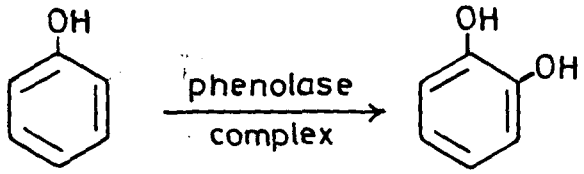
3. PHENOLASE COMPLEX

Phenolase complex (Tyrosinase) can catalyse two types of oxidations:

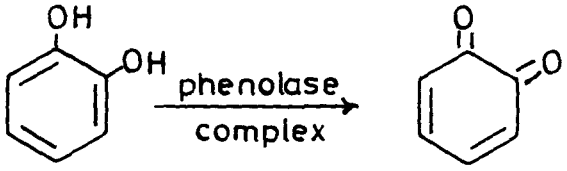
(a) o-hydroxylation of monohydric phenols to form dihydroxy compound (Eq. V)

(b) Oxidation of dihydric phenols to corresponding quinones (Eq. VI).

Equation V:



Equation VI:



The occurrence of first reaction differentiates tyrosinase sharply from laccase. The second reaction, involv oxidation and coupling of polyhydric phenols.

(i) Occurrence - Phenolase complex (tyrosinase) is widely distributed in nature, but its presence in mammals has not be so easy to demonstrate. Onslow (63) reported its extraction from the skin of rabbits of various coloured races. Gessard (64) showed the presence of the enzyme in cuttlefish. Pihey (65) reported its presence in the blood of certain Crustaceae Bhagvat and Richter (66) found evidence for the presence of the enzyme in the blood of arthropods. More complete account of their occurrence in plants (67), in wood rotting fungi (38) and in mammalian tissue (68) have already been reviewed. Care must be excercised, however, to interpret the data as many of the investigations cited do not differentiate clearly between phenolase complex and laccase.

The sources mostly used for obtaining preparations of phenolase complex and for the study of the enzyme and it

action have been the potato (*solanum tuberosum*) and common mushroom (*Psalliota campestris*). The larvae of the meal wor (*Tenebrio molitor*), the wild mushroom (*Lactarius piperatus*) (69), the blood of the octopus (70), the Indian bean (71) and wheat bran (72) have also been used.

(ii) *Physico-chemical Characteristics* - The conclusions dr from many investigations on partially purified phenolase complex (tyrosinase) preparations have been summarized in Table III (69,73,74,75). It should be however, pointed out that purification of tyrosinases is a very difficult task, as they usually occur in tissue in very low concentrations together with potential substrates whose removal is necessar during purification which results in heavy losses of the enzyme.

Phenolase complex consists of protein coordinated to copper and no coenzyme appears to be required for the activity. The copper is firmly bound to the protein but can be removed by dialysis against 0.01M HCN, followed by dialysis against phosphate buffer.

Table III

Properties of Phenolase Complex

Source	Molecular weight	Cofactor	λMax
			(m μ)
White potato (*Solanum tuberosum*)	100,000	Cu^{++} (?)	275
Common edible mushroom (*Agaricus campestris*)	-	Cu^{++}	-
Wild mushroom (*Lactarius piperitus*)	-	Cu^{++}	278,330
Common mushroom (*Psalliota campestris*)	128,000	Cu^{++}	282 with shoulder at 290

One of the most striking characteristics of oxidatic of catechol by phenolase complex is the pronounced inactivation or destruction of the enzyme that occurs durir the course of reaction. The enzyme inactivation is so readily apparent during the course of the reaction that mar investigators have concerned themselves with the problem. Because of the character of inactivation it seems at first logical to attribute it to the destructive action of the products formed during the course of oxidation. Ludwig and Nelson (76), however, conclusively demonstrated that the inactivation is not due to o-benzoquinone or any of its polymerization products. Keeping in view that all known products of oxidation of catechol have been eliminated in this consideration, it would appear that inactivation occur as a direct result of the catalytic action of the enzyme ar is due to some factors inherent in the substrate-enzyme-oxygen system.

During the aerobic oxidation of catechol it is usually observed that the rate of oxygen consumption falls off rapidly from the moment the reaction is started and

unless relatively large amounts of enzyme are employed initially, the oxidation stops before the catechol is completely oxidised. This is due to rapid inactivation of enzyme during the reaction. It is therefore difficult to obtain reliable measurements of initial rate of oxidation. Different investigators have attempted to get around this problem in following ways.

From the quantitative view point the earlier colorimetric methods of enzyme assay were not very satisfacto and have been largely replaced by manometric methods. The rate of pigmentation during the enzymic oxidation is not necessarily proportional to the rate of oxidation or to the amount of enzyme. The pigment development is markedly influenced by factors such as pH, the presence of amino acids the presence of reducing agents such as ascorbic acid. It must be pointed out that if any significant amount of reducin material such as ascorbic acid is also present in the plant tissue, there is likely to be no colour development for somet even though the tissue contains appreciable amount of phenola

Graubard and Nelson (77) used the volume of oxygen absorbed in sixty minutes as a measure of enzyme activity. B Nelson and Dawson (78) showed that a unit of enzyme based on the volume of oxygen absorbed during the inactivation process varies in magnitude from one preparation to the other, dependi largely on the method of isolation and purification.

Gregg and Nelson (79) used gelatin in the incubation mixture to protect the enzyme against inactivation. It was observed by Miller and Dawson (80) that the protective action of gelatin was variable and depended on a number of factors. Keilin and Mann (74) made no statement regarding the possible error in their measurements, but it is to be noted that they used very large amounts of enzyme for determination.

Kubowitz (73) estimated the activity of his potato oxidase preparation by determining the rate of aerobic oxidation ofhexosemonophosphate in the presence of its dehydrogenase, NADP and a very small amount of catechol. Kubowitz showed that the rate of oxygen uptake was proportional to the enzyme concentration for twenty minutes. Probably because of the complexity of the system and because of the fact that

oxygen uptake was dependant on other factors in the system tha the enzyme, and it involves agents which are not always easily available, Kubowitz method of measuring activity has not found wide application.

Adams and Nelson (81) added hydroquinone and gelatin in the incubation mixture and obtained linear reaction course. More recent studies have shown that hydroquinone does not act merely as a reductant for the o-benzoquinone but under certain conditions it is oxidized by the enzyme (82). These effects vary widely under different conditions (80,83).

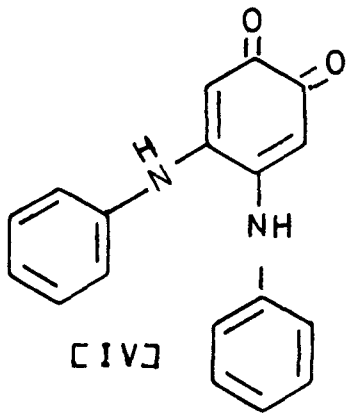
Nelson and Dawson (78) first pointed out that reliable activity measurements should be based on initial reaction velocity. The lack of precision inherent in manometric methods, when attempts are made to take readings of oxygen uptake within the first two or three minutes, makes determination of initial rate impracticable. It is possible, however, to follow with good precision the production of o-benzoquinone during the early stages of reaction. Nelson and Dawson therefore developed a new method, called chronometric method, since it involves a measurement of time required for a given quantity

of enzyme to produce o-benzoquinone just in excess to that necessary to oxidise a small amount of ascorbic acid also present in the system. The authors have pointed out that in their system ascorbic acid was not directly oxidised by the enzyme nor it effects inactivation characteristic of the enzyme. This method is likely to give erroneous results in the system where ascorbic acid is directly oxidised by ascorbic acid oxidase present therein. In such a case the only choice remains that of manometric measurements of oxygen consumption, although there is all the likelyhood of the possible error.

(iii) Mechanism - Phenolase complexes are capable of catalyzing the oxidation of mono as well as o-polyhydric-phenols . The mechanism of this process is at present obscure. It would appear that aryloxy radicals must be produced, which leads to the conclusion that copper is acting in cupric state. There are differences between the action of phenolase complex and that of laccase, which points a difference in mechanism. From practical as well as mechanistic point of view, differentiation of phenolase complex from laccase is important (Table IV). Some of the tests listed in the table IV should be applied in

all work on multiplicity and fractionation of phenol oxidases.

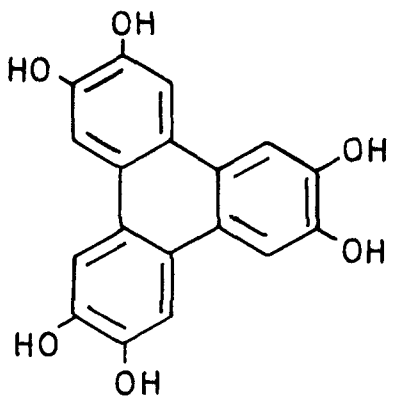
(iv) Products - Phenolase complex catalyzed oxidation of catechol also yeilds o-benzoquinone as does laccase catalyzed reaction. Pough and Raper (62) carried out the oxidation in th presence of aniline and isolated dianilino-o-benzoquinone.



[IV]

(dianilino-o-benzoquinone)

Piattelli et.al (84) have suggested that the structures of the type (V) could arise by oxidative coupling of catechol. In support of this view Nicolaus and Piattelli (85) have shown that phenolase catalyzes the oxidation of this compound.



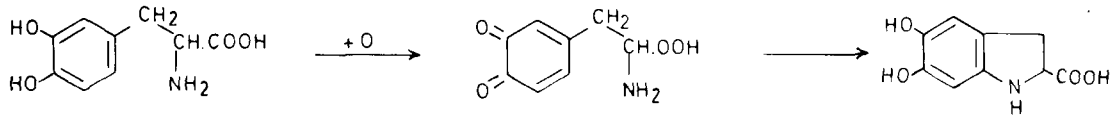
[V]

Table IV

Differences Between Phenolase Complex and Laccases

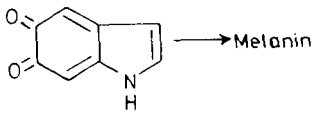
Property	Phenolase complex	Laccases
State of Copper	Cu^{++}	Cu^{++}
Ultaviolet absorption	280 mμ	280 mμ, 615 mμ
Occurrence of hydroxylation reaction	+	-
Inhibition by carbon monoxide	+	-
Hydroquinone as substrate	No action	p-benzoquinone produced
Tyrosine as substrate	coloured products	No action
p-Cresol as substrate	Coloured products	Colourless products
Guaiacol as substrate	No action	Coloured products

Fig. 1. Pathway for melanin biosynthesis.



DOPA DOPA QUINONE LEUCO COMPOUND

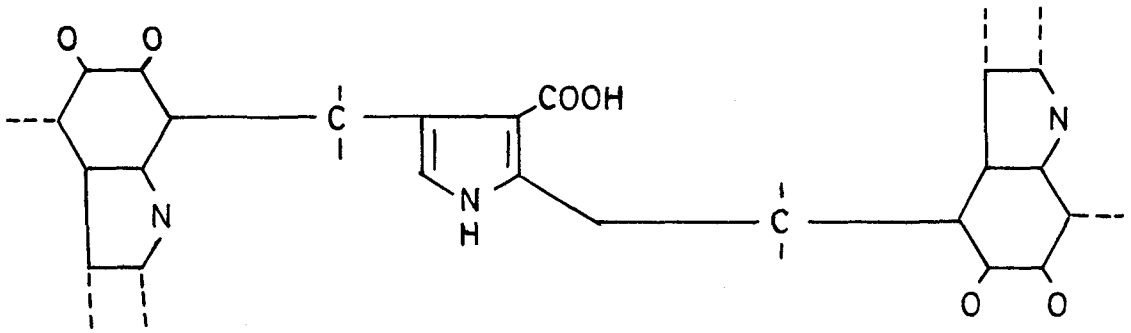
DOPACHROME 5,6- DIHYDROXYINDOLE



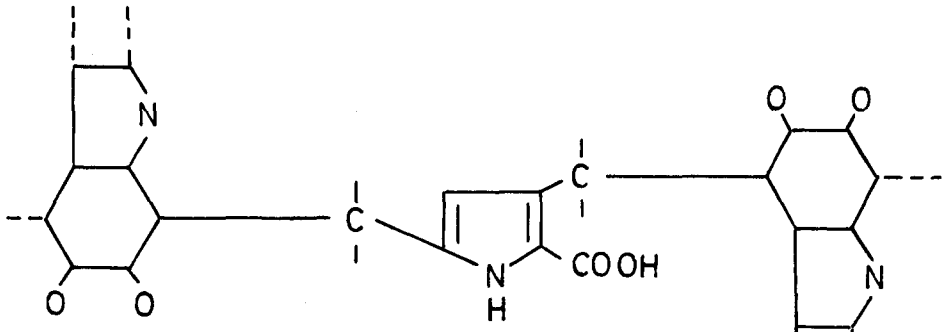
INDOLE 5,6-QUINONE

weight polymer. Melanin with its reactive groups can combine with proteins to produce the dark pigments.

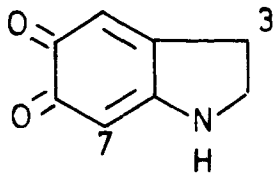
Investigations on the structure of sepiomelanin a nitrogeneous melanin from cuttlefish ink, have shown (88) that some structures of the type VI, VII and VIII are present.

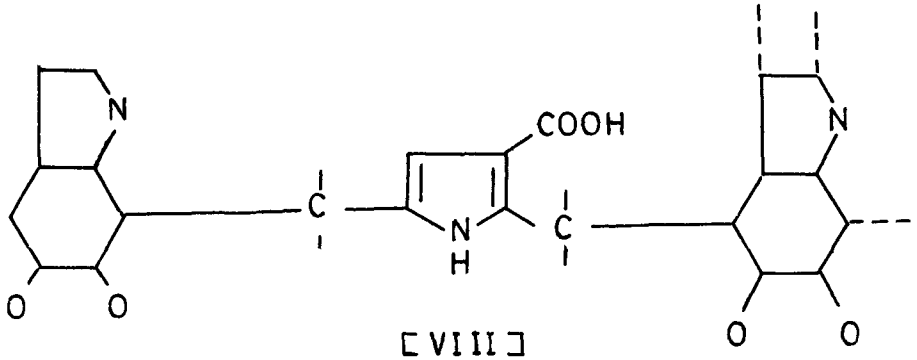


[VI]



[VII]





[VIII]

V. MATERIALS AND METHODS

1. Chemicals - The following chemicals were obtained commercially and used without further purification : 3,4-dihydroxy-phenylalanine (DOPA), 3,4-dihydroxy-phenyl-ethylamine (DOPAMINE threamine, nor-leucamine, B-phenylethylamine, tyramine, seramine cadaverine, putrescine, 3-methoxy-4-hydroxy-phenyl-ethylamine, valamine, histamine, tryptamine, leucamine, tyrosine and phenylalanine were purchased from Calbiochem, U.S.A. Catalase was obained from Sigma Chemicals Co., U.S.A. Bovine serum albumin was purchased from Mann Research Laboratories, U.S.A., in crystalline form. Rest of the chemicals used were of A.R. grade.

2. Plant Materials - The plants were either grown in the garden of the department or in the wooden pots.

3. Enzyme Preparations -

(i) Crude Homogenate (IO %, w/v) - The plant material washed free from soil was accurately weighed and transferred to a chilled Potter-Elvehjem all glass homogenizer containing 0.02M

sodium phosphate buffer, pH 7.0. The homogenization was carried out for 15 minutes. The homogenate was squeezed through two layers of muslin cloth and centrifuged at 1,200 x g for 15 minutes to remove cell debris. The supernatant thus obtained was used as a source of crude enzyme.

(ii) Acetone Powder Extracts (10%, w/v) - Acetone dried powders were prepared by placing the cut plant in a chilled waring blender, covering with 5 volumes of cold acetone (-10°) and blending vigorously for one minute. The resulting slurry was quickly filtered with suction through the Buchner funnel, the residue spread out on filter paper, dried at room temperature and sifted through wire mesh. The acetone dried powder thus obtained was stored in a desiccator at 4°.

Acetone powder of whole plant (10%, w/v) was suspended in 0.02 M sodium phosphate buffer, pH 7.0. The suspension was mechanically stirred for half an hour at 5° and then squeezed through two layers of muslin cloth. The extract obtained was centrifuged at 10,000 x g for 15 minutes to remove floating particles. The suspension was used as a source of enzyme.

4. Manometric Studies - Manometric experiments were carried out at 30° in a conventional Warburg apparatus using single or double arm flasks shaken at 105 strokes per minute (89).

For oxygen uptake studies, the center well contained 0.2 ml of 20 % KOH and a small roll of filter paper. For following carbon dioxide evolutin and concurrent oxygen utilization, KOH was omitted from the center well and the indirect method of Warburg was used. In all the cases, unless indicated otherwise, air was used as gas phase. The rate of oxidation as well as carbon dioxide evolution has been calculated from the initial rate. One enzyme unit is defined as the amount of enzyme which is responsible for the consumption of one microliter of oxygen per hour. The specific activity has been expressed in terms of enzyme units per mg protein.

5. Estimation of Ammonia - This was estimated by the modified aeration method of Van Slyke and Cullen. In the original procedure the aerated ammonia is absorbed in standard acid and the acid back titrated with standard alkali. In the modificati

described here, the ammonia is absorbed in boric acid solution and titrated directly with standard acid (90).

2 ml ofincubation mixture was placed in one of the two large test tubes used in the aeration train. The test tube was connected for aeration with a second tube containing 25 ml of 2% boric acid containing bromocresol green indicator. The stopper of the tube containing the incubation mixture was removed and 5 ml of saturated potassium carbonate solution adde The stopper was placed tightly and the air current flown. The incoming air was washed by preliminary passage through a simila aeration train test tube containing 10% sulfuric acid to remove any ammonia present. After one hour of aeration, the tube containing boric acid solution was removed, the inlet tube rins and the contents were titrated with 0.01 N sulfuric acid until blue colour, if any, is replaced by original yellow green colou as determined by matching against a control (25 ml portion of boric acid indicator diluted with water to approximately the final volume of the titrated sample).

Boric acid solution containing bromocresol green indicator was prepared by dissolving 20 gm of boric acid in 50C

of hot water. 2 ml of 0.1% bromocresol green in alcohol was added to the cooled boric acid solution and diluted to one liter.

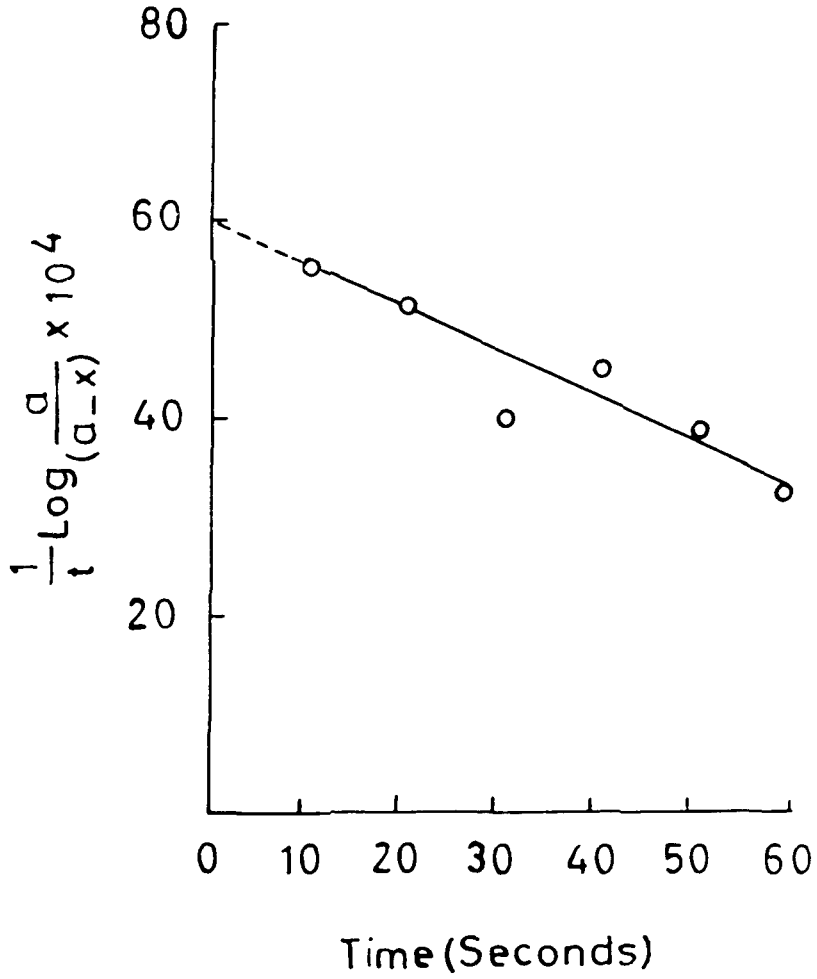
6. Estimation of Catalase Activity - Catalase activity was estimated by potassium permanganate titration method (92).

At a temperature of 20°, 15 ml of 0.01 M hydrogen peroxide in 0.01 M sodium phosphate buffer, pH 7.0, was placed in an Erlenmeyer flask. For zero time reading, 2.0 ml of this solution was withdrawn and added to 5 ml of 2% sulfuric acid for titration with 0.01 N potassium permanganate solution. 0.1 ml of 1 : 1000 diluted catalase was pipetted into the peroxide solution which was being swirled rapidly. Samples of 2 ml each were rapidly withdrawn at 10, 20, 30, 40, 50 and 60 seconds and blown out into seperate flasks containing 5 ml of 2% sulfuric acid. From the equation:

$$K = \frac{I}{t} \log_{10} \frac{a}{(a - x)}$$

where a is the initial hydrogen peroxide concentration and

Fig. 2. Monomolecular velocity constants Vs time plot for calculation of Kat.f. value of catalase. See text for details.


80
60
40
20
$\frac{1}{t}\text{Log}\frac{a}{(a-x)} \times 10^4$
0
10
20
30
40
50
60
Time (Seconds)

(a - x) is the concentration at time t, k values for various time intervals were calculated and K value at zero time obtained by extrapolation (Fig.2). Using this value for K, the Katalasefahigkeit (Kat. f.)

$$\text{Kat. f.} = \frac{K}{\text{g. enzyme}}$$

was found to be 2,30,700.

7. Estimation of Hydrogen Peroxide - Hydrogen peroxide formation was tested by method of Tabor (91). The decrease in oxygen uptake in presence of initially added catalase was used as a measure of hydrogen peroxide formation.

8. Estimation of Quinones - The method employed was essentially the same as that of Dawson and Nelson (93). It is based on the principle that iodine set free from potassium iodide by o-benzoquinone formed during the reaction is proportional to

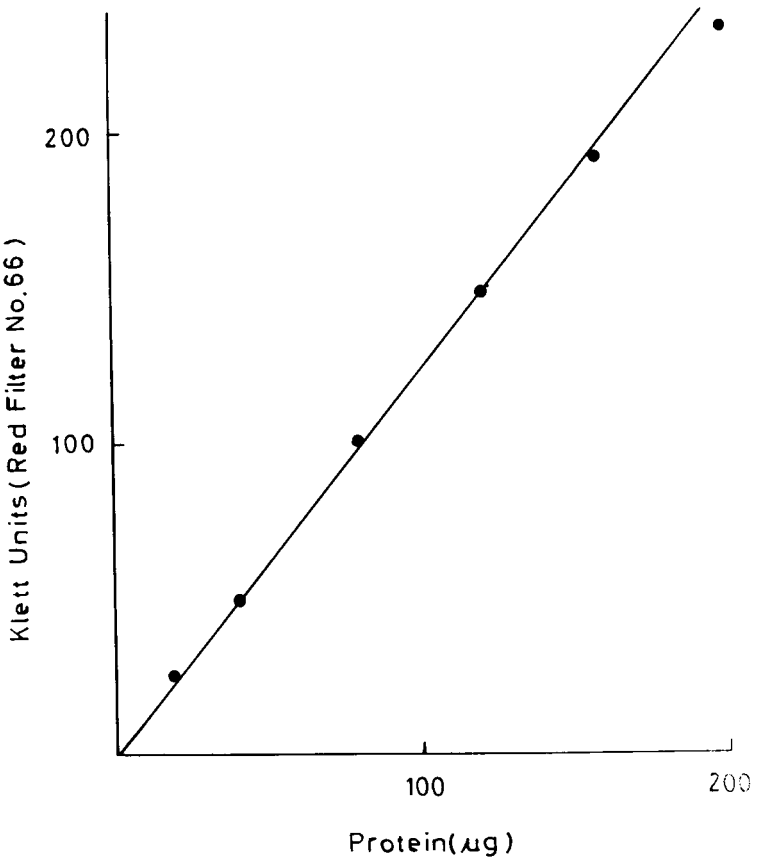
the amount of quinone and could be titrated with standard sodium thiosulfate solution.

To the 2 ml of incubation mixture was added, 0.5 ml of 10% sulfuric acid and 1 ml of 10% potassium iodide. The reaction mixute was allowed to stand in dark for 15 minutes. Four drops of starch were added and the blue colour was titrated against standard 0.0085 N sodium thiosulfate solution. 1 ml of 0.0085 N sodium thiosulfate is equivalent to 4.25 umoles of quinone formed during the reaction.

9. Estimation of Protein - Protein was estimated by Folin and Ciocaulteu method as described by Lowry et. al. (94).

To 1 ml sample containing suitable quantity of protein, 5.0 ml of alkaline copper reagent (1 ml of 0.5% copper sulfate in 1% potassium tartrate solution added to 50 ml of 2% sodium carbonate in 0.1 N NaOH solution) was added and incubated at room temperature for 15 minutes. Folin reagent (1 N) was added, mixed instantly and colour density measured after 30

Fig. 3. Standard curve for protein estimation. See text for details.


200
100
Klett Units (Red Filter No.66)
100
200
Protein(μg)

minutes in a Klett-Summerson colorimeter using red filter against the reagent blank. Crystalline bovine serum albumin solution was used as standard (Fig. 3). Under these conditions one Klett unit was found to be equal to 0.805 ug of standard protein.

10. Preparation of Carboxymethyl Cellulose Column - CM-cellulose was suspended in distilled water and packed uniformly in a column (1.2 x 15 cm) without air bubbles getting trapped. 200 ml of 1 N HCl was passed through the column and excess of acid washed with glass distilled water till the washings were free of acid . 200 ml of 1 N NaOH is then passed through the column. After removing excess of alkali with distilled water, the column was finally washed and equilibrated with 0.01 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0.

11. Preparation of Diethylaminoethyl Cellulose Column - DEAE-cellulose was suspended in 1 N NaOH taking care to avoid air

bubbles. The pale yellow supernatant was poured off and resin washed with distilled water. The resin was packed in a column (1 x 15 cm) without air bubbles getting trapped. The column was washed with large volumes of glass distilled water till the washings were free of alkali. The chloride form of the resin was obtained by passing 200 ml of 1 N HCl through the column and washing off the excess of acid with glass distilled water. The column was then equilibrated with 0.01 M sodium phosphate buffer, pH 7.0.

VI. RESULTS

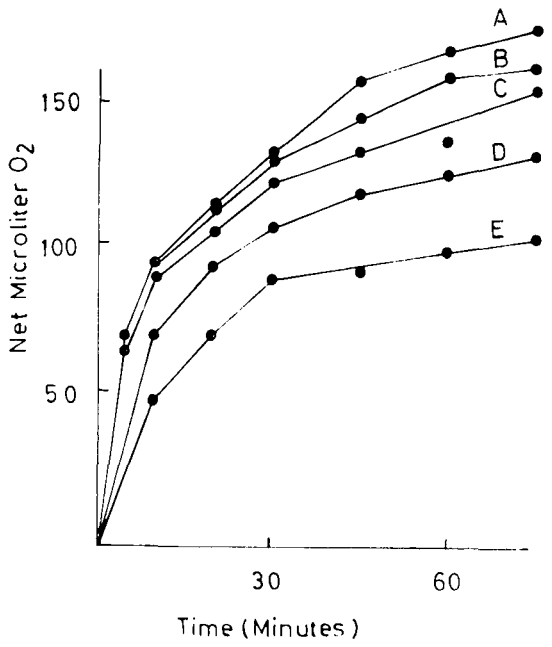
1. Oxidation of DOPA by the Extracts of Papaver somniferum - The roots, leaves, stems, fruits and buds or whole seedlings of P.somniferum are capable of oxidising DOPA. The rate of oxygen uptake by the extracts of these tissues was, however, linear for a very short time (Figs. 4 & 5). These extracts did not oxidise either tyrosine or phenylalanine when tested over a wide range of pH (pH 5 - 9). The DOPA oxidising ability was retained by the acetone dried powders of whole plant. The oxidation of DOPA by the plant extracts suggested the presence of amino acid oxidase, amine oxidase or phenolase complex in P.somniferum. From the initial rate of oxidation, it may be readily calculated that there is not much difference in the specific activity of the enxyme oxidising DOPA in the various tissues of the plants. However seedlings seem to contain much higher activity as compared to mature plant.

2. Carbon Dioxide Evolution by the Extracts of P.somniferum in Presence of DOPA - Rapid evolution of carbon dioxide, by the crude extracts of roots, leaves, stems, buds or whole see ing extracts in presence of DOPA, was observed (Figs. 6 & 7).

Fig. 4. Oxidation of DOPA by the extracts of P.somniferum. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 40 µmoles of sodium phosphate buffer, pH 7.0, and enzyme protein. The side arm contained 4 µmoles of DOPA in 0.2 ml.

A.	Fruits	(611 µg. protein)
B.	Buds	(785 µg. protein)
C.	Leaves	(1151 µg protein)
D.	Roots	(556 µg protein)
E.	Stems	(379 µg protein)

Fig. 5. Oxidation of DOPA by extracts of P.somniferum seedlings. The incubation mixture was the same as described for Fig. 4, except that seedlings extracts equivalent to 176 µg of protein was used as enzyme.


A
B
C
D
E
150
100
50
Net Microliter O_2
30
60
Time (Minutes)

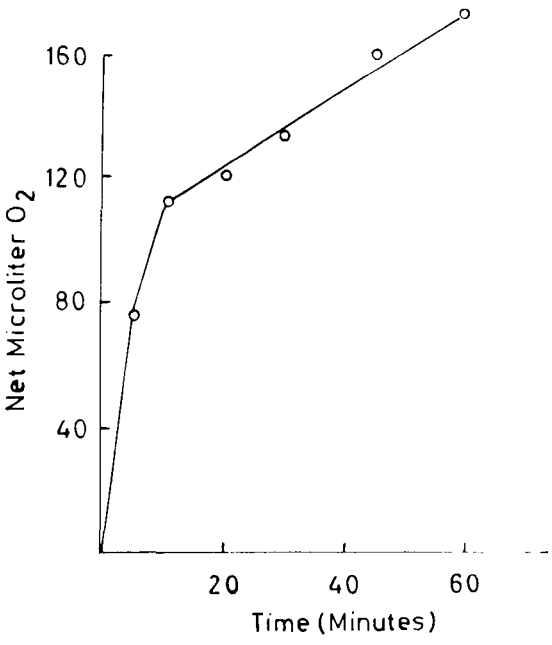
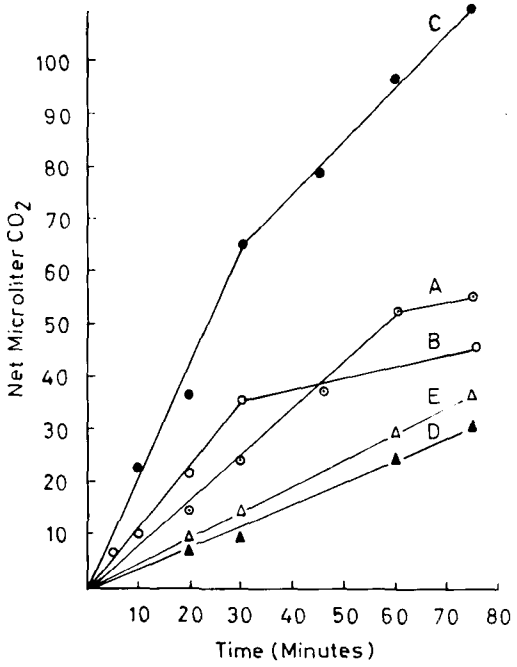
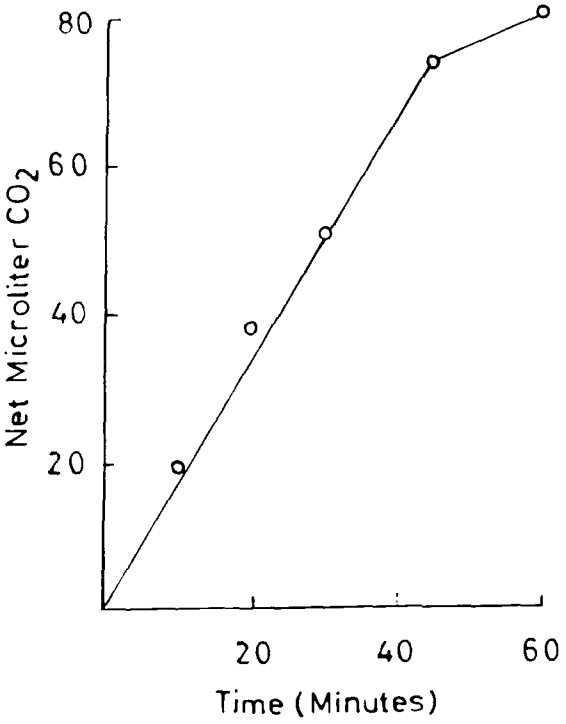

160
120
80
40
Net Microliter O_2
20
40
60
Time (Minutes)

Fig. 6. Carbon dioxide evolution by the extracts of P.somniferum in presence of DOPA. The incubation mixture was the same as described for Fig. 4, except that appropriate flasks containing no KOH in the center well were also included.

Fig. 7. Carbon dioxide evolution by the extracts of P.somniferum seedlings in presence of DOPA. The incubation mixture was the same as described for Fig. 5, except that appropriate flasks containing no KOH in the center well were also included.


C
A
B
E
D
100
90
80
70
60
50
40
30
20
10
Net Microliter CO_2
10 20 30 40 50 60 70 80
Time (Minutes)

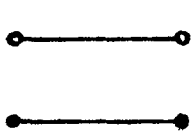

80
60
40
20
Net Microliter CO_2
20 40 60
Time (Minutes)

Under the assay conditions, the rate of carbon dioxide evolution was found linear for 20 to 30 minutes. It is apparent from the rate of carbon dioxide evolution that in all the tissues tested, seedlings appear to contain highest specific activity.

The evolution of carbon dioxide by the extracts in presence of DOPA suggested the possibility of the presence of DOPA decarboxylase in P.somniferum. But since this decarboxylation was accompanied with the oxidation of DOPA, it was also possible that first DOPA is oxidised and the resulting product is then decarboxylated. Using acetone power extracts of whole plant it has been found that the decarboxylation of DOPA was completely inhibited under nitrogen atmosphere (Fig. 8), thereby the carbon dioxide evolution in presence of crude homogenate or acetone powder extract may not be due to the decarboxylation of DOPA itself but it could mean decarboxylation of some oxidation product of DOPA.

3. Oxidation of Various Amines by the Extracts of P.sombiferum - Table V shows the increased oxygen uptake by the seedlings extract of P.somniferum in presence of various amines. The

Fig. 8. Carbon dioxide evolution in inert atmosphere. The main compartment of Warburg flask, in a total volume of 2.5 ml, contained 250 umoles of sodium phosphate buffer, pH 7.0, and acetone powder extract equivalent to 6.5 mg of protein. The side arm contained 4 μmoles of DOPA in 0.5 ml. Appropriate flasks containing no KOH in the central well were included. Another similar set of flasks was run simultaneously with nitrogen as gas phase instead of air.



Air phase

Nitrogen phase

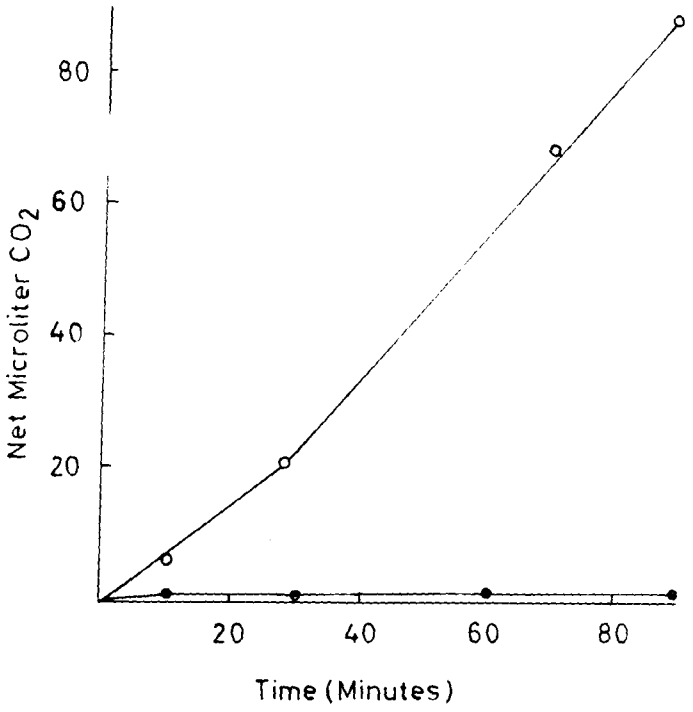

80
60
40
20
Net Microliter CO_2
20
40
60
80
Time (Minutes)

Table V

Oxidation of Various Amines by the Extracts of P.somniferum

The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 40 µmoles of sodium phosphate buffer, pH 7.0, and 163 µg of enzyme protein. The side arm contained 4 µmoles of substrate, as indicated , in 0.2 ml.

Substrate	Increased O_2 uptake (µl/hour)	Substrate	Increased O_2 uptake (µl/hour)
DOPAMINE	114	nor-leucamine	0
Tyramine	17	Valamine	0
DOPA	62	Seramine	0
Tyrosine	0	Threamine	0
3-methoxy-4-hydroxy phenylethylamine	0	Cadaverine Putrescine	0 0
β-Phenylethylamine	0	Histamine	<5
Leucamine	0	Tryptamine	<5
2-2-dithio-bis-(ethylamine)	<5	Phenylalanine	0

results are presented in terms of increased oxygen uptake per hour rather than as velocities, because tyramine is oxidised with lag phase. It is apparent from the data that none of the amines except those which contain hydroxyl group in the aromatic nucleus are oxidised by the extracts of P.somniferum. Cadaverine and putrescine, the best substrates of amine oxidase (95) are not at all oxidised even with as concentrated enzyme as 50% leaf homogenate. Furthermore, the oxidation of DOPAMINE and tyramine was not accompanied with ammonia and hydrogen peroxide formation (Table VI). No decrease in oxygen uptake could even be noted when catalase was added initially in the incubation mixture suggesting that no hydrogen peroxide was formed during the oxidation of these substrates (Fig. 9). Similar results were obtained with acetone powder extracts.

4. Oxidation of Various Phenols by the Extracts of P.somniferum - As shown earlier, plant extracts were unable to oxidise phenylalanine, tyrosine, cadaverine and putrescine. Furthermore, the oxidation of DOPAMINE and tyramine was not

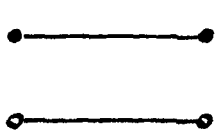
Table VI

Oxygen Uptake, Ammonia and Hydrogen Peroxide Formation During the Oxidation of DOPAMINE and Tyramine

For oxygen uptake studies, the incubation mixture was the same as described for Fig. 9 except that no catalase was added. Similar mixture was incubated seperately for one hour for ammonia estimation as described in the text. For hydrogen peroxide formation, see Fig. 9.

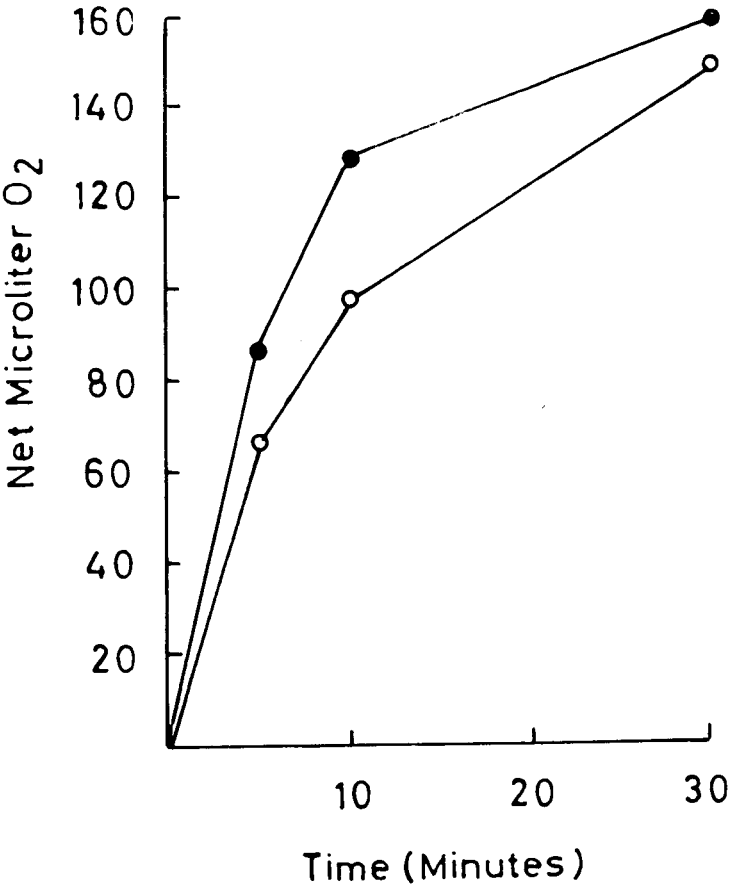
Substrate	Increased O_2 uptake	Ammonia	Hydrogen peroxide
	(µl/hour)	(µmoles)	(µmoles)
DOPAMINE	310	0	0
Tyramine	167	0	0

Fig. 9. Effect of catalase on oxygen uptake. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 80 µmoles of sodium phosphate buffer, pH 7.0, 400 µg catalase and 140 µg of 32-fold purified enzyme preparation. (see Table XVI) The side arm contained 10 µmoles of DOPAMINE in 0.2 ml.



Catalase present

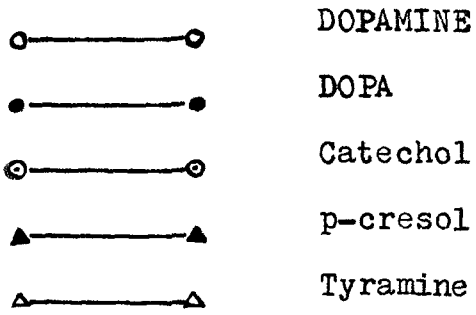
No catalase


160
140
120
100
80
60
40
20
Net Microliter O_2
10
20
30
Time (Minutes)

accompanied by ammonia or hydrogen peroxide formation. Therefore it was concluded that DOPA, DOPAMINE and tyramine oxidising ability of plant extracts was not due to amino acid oxidase or amine oxidase but due to phenolase complex activity.

To test this possibility, the ability of P.somniferum seedling extracts to oxidise various other phenolic compounds was studied. As shown in the Table VII, o-diphenols like DOPA, DOPAMINE and catechol were most readily oxidised whereas the oxidation of monophenols such as p-cresol and tyramine was relatively slow and exhibited a lag phase (Fig. 10). However, m-cresol was not oxidised. Tyrosine was not oxidised even in presence of added catechol (Table VIII). These results suggested the presence of a distinct phenolase complex in P.somniferum. It is interesting to note that with DOPA, DOPAMINE and tyramine as substrates a black product is formed while with p-cresol, and catechol red product is formed in the reaction mixture.

Fig. 10. Oxidation of various phenols by the extracts of P.somniferum seedlings. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 40 µmoles of sodium phosphate buffer, pH 7.0, and seedling extract equivalent to 156 µg of protein. The side arm contained 4 µmoles of substrate in 0.2 ml.



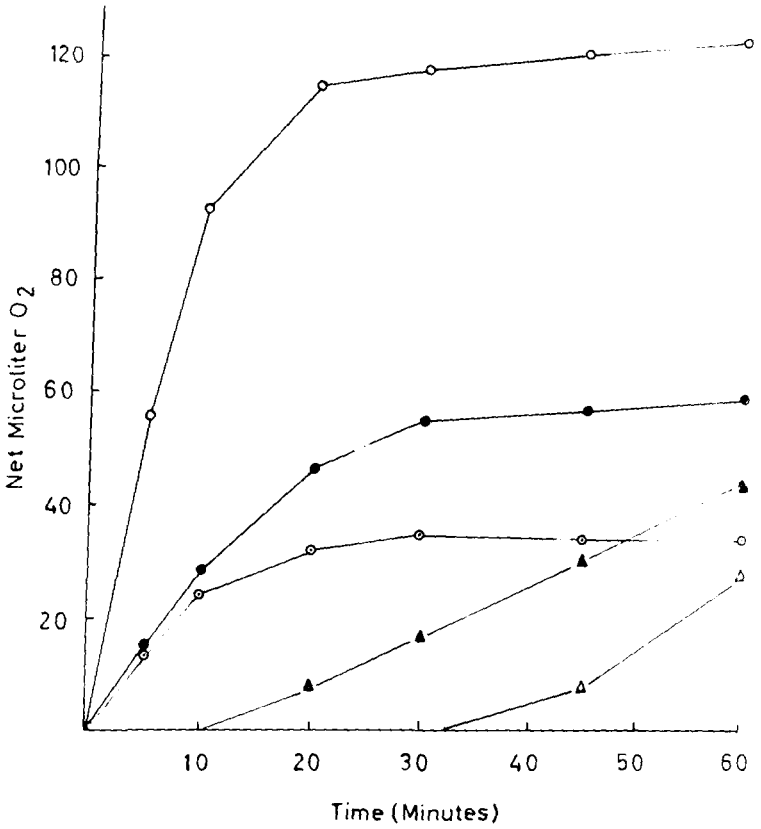

120
100
80
60
40
20
Net Microliter O_2
10
20
30
40
50
60
Time (Minutes)

Table VII

Oxidation of Various Phenols by the Extracts of P.somniferum

The incubation mixture was similar to that described for Fig. 10.

Substrate	Increased O_2 uptake (µl/hour)
DOPAMINE	127
DOPA	61
Catechol	35
Tyramine	28
p-cresol	45
Tyrosine	0
Hydroquinone	0
5-hydroxytryptophan	0
3-hydroxyanthrinilic acid	0
vanillin	0
p-phenylenediamine	0
m-cresol	0

Table VIII

Effect of Catechol on Tyrosine Oxidation

The main compartment of Warburg flask, in a total volume of 1.6 ml, contained 100 µmoles of sodium phosphate buffer, pH 7.0, and acetone powder extract equivalent to 520 µg protein. One of the side arms contained 4 µmoles of tyrosine in 0.2 ml while the second contained 4 µmoles of catechol in 0.2 ml. The center well contained 0.2 ml of 20% KOH. After equilibration for 10 minutes, the contents of the both side arms were simultaneously tipped into the main compartment of the flask. Suitable controls were included to correct for endogeneous uptake of oxygen.

System	Increased O_2 uptake
	(µl/hour)
Extract	0
Extract + tyrosine	0
Extract + catechol	40
Extract + catechol + tyrosine	40

5. Variation of Phenolase Complex Activity with Age of the Seedlings - The correlation of pehnolase complex activity towards dopamine with age of P.somniferum seedlings has been investigated. At various stages of growth the phenolase activity was determined. A typical set of results is given in Table IX. It is evident that the activity increases over a period of 15 to 19 days and then decreases upto 27th day when again a rise in activity was observed which reached the peak value in eighty days. After this stage of growth, it was not possible to use entire seedlings as the plant showed well differentiated roots, stems and leaves.

6. Distribution of Activity - At later stages of growth, when sufficient leaves had developed to enable differentiation between stem, leaves and buds, activity was found to be distributed in all the tissues tested (Table X). The activity was found progressively increasing from roots to buds. The activity within these tissues also showed variation with age. It is interesting to note that the activity of buds as well as unfertilized fruits was highest as compared to the other tissues and

Table IX

Variation on Phenolase Complex Activity with Age of the Seedlings

The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 40 µmoles of sodium phosphate buffer, pH 7.0, and 0.2 ml seedling extract. The side arm contained 4 µmoles of DOPAMINE in 0.2 ml.

Age	Specific Activity
(days)	(µl O_2/hr./mg protein)
8	3246
11	3532
15	4439
19	4443
23	2619
27	1574
40	2892
54	2753
67	3533
79	4279

Table X

Distribution of Phenolase Complex Activity in Various Tissues of Papaver somniferum

The incubation mixture was similar to that described for Table IX.

Tissue	Specific Activity ($\mu l/O_2/hr./mg$ protein		
	107 days	112 days	119 days
Roots	3570	4950	5590
Stems	3838	5666	5000
Leaves	7432	7092	5386
Buds	15452	-	12886
Fruits (unfertilized)	-	1386	-
Fruits (fertilized)	-	-	14462

seedlings. In light of these observations, the progressive increase in phenolase complex activity of P.somniferum with age of the plant and maximum activity in the buds and fruits may be attributed to the protective coating and functional as well as adventitious browning capacity of the plant (96).

7. Kinetic Studies - In order to gain more insight regarding the nature of P.somniferum phenolase complex, detailed kinetic studies were undertaken.

(i) Effect of Substrate Concentration - The effect of varying concentrations of DOPAMINE and tyramine on the oxygen uptake was investigated. The results are shown in the Figs. 11 & 12. In case of DOPAMINE as substrate, the substrate concentration is plotted against initial reaction velocity whereas in case of tyramine oxidation, the substrate concentration is plotted against increased oxygen uptake because of the observed lag phase. In case of tyramine, higher concentrations were inhibitory.

Fig. 11. Effect of DOPAMINE concentration on phenolase complex activity of P.somniferum. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 100 µmoles of sodium phosphate buffer, pH 7.0, and acetone powder extract equivalent to 520 µg protein. The side arm contained varying concentrations of DOPAMINE in 0.2 ml.

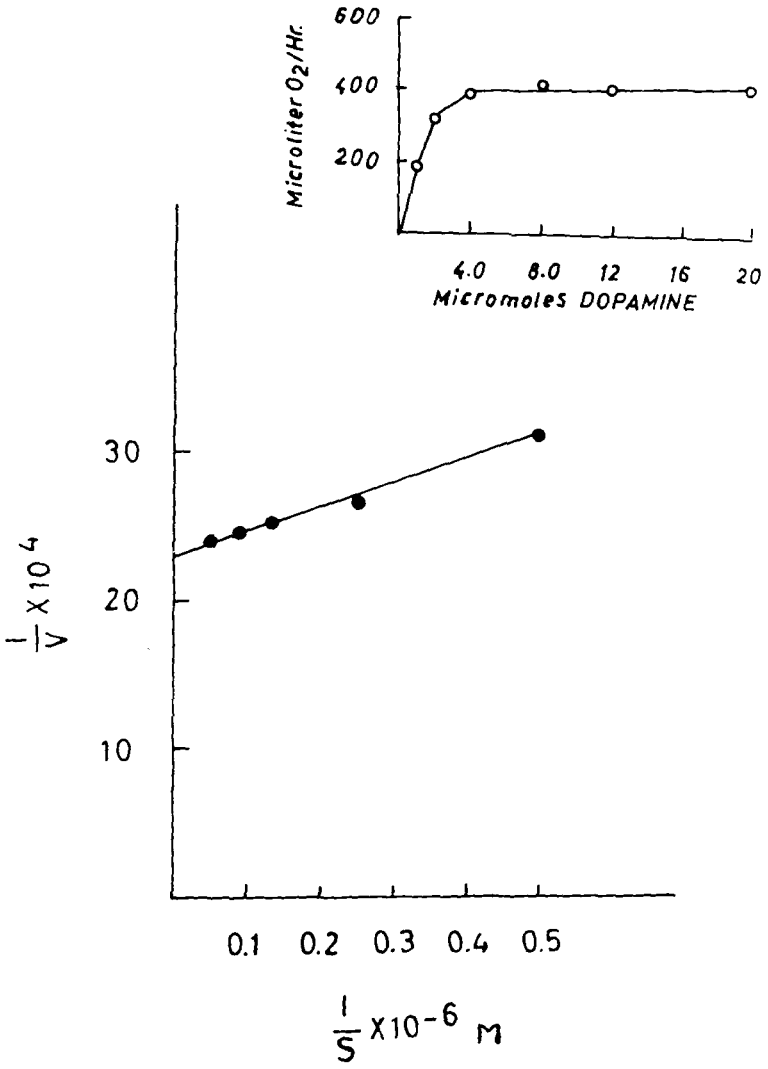
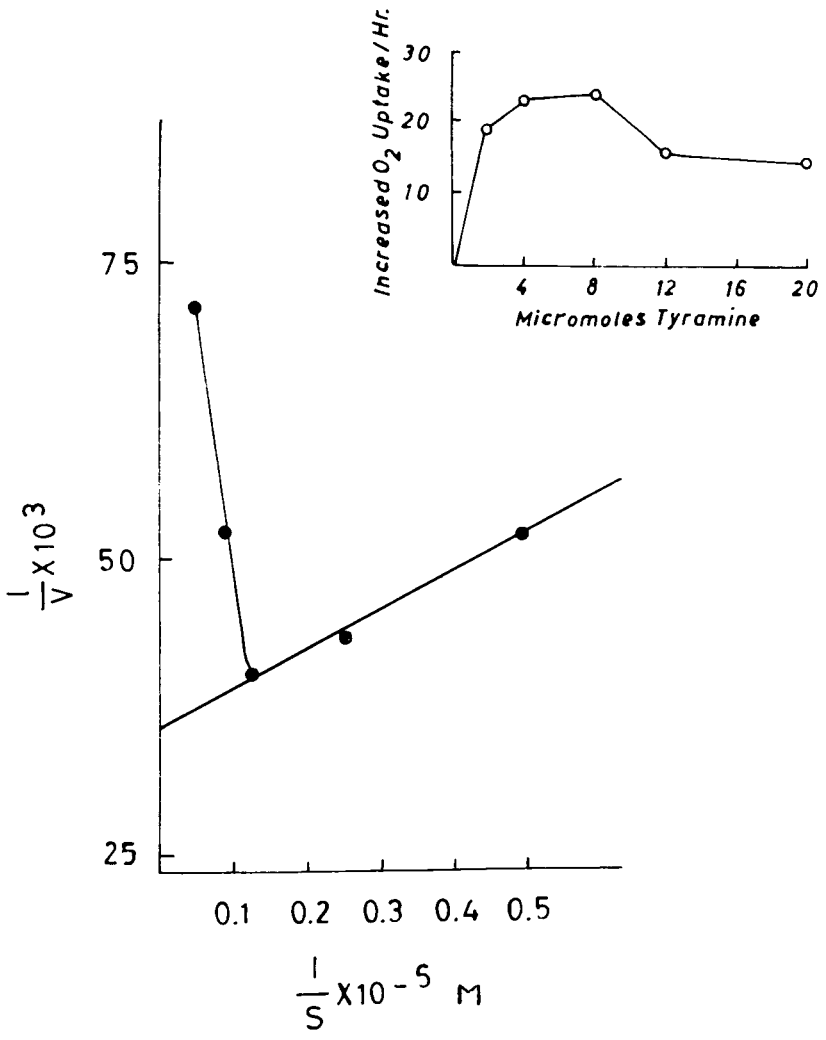

600
400
200
Microliter O_2/Hr.
4.0
8.0
12
16
20
Micromoles DOPAMINE
30
20
10
$\frac{1}{V} \times 10^4$
0.1
0.2
0.3
0.4
0.5
$\frac{1}{S} \times 10^{-6}$ M

Fig. 12. Effect of tyramine concentration on phenolase complex activity of P. somniferum. The incubation mixture was the same as described for Fig. 11 except that tyramine was used as substrate.


Increased O_2 Uptake / Hr.
30
20
10
4
8
12
16
20
Micromoles Tyramine
75
50
25
$\frac{1}{V} \times 10^3$
0.1
0.2
0.3
0.4
0.5
$\frac{1}{S} \times 10^{-5}$ M

The Km values calculated from the graphs are $1.7X10^{-6}$ M for DOPAMINE oxidation and $1.0X10^{-6}$ M for tyramine oxidation.

(ii) Effect of pH - Figs. 13 and 14 show the variation of phenolase complex activity with the change of pH using DOPAMINE and tyramine as substrates. Since with tyramine an initial lag phase was observed, the increased oxygen uptake in 45 minutes has been plotted against pH (Fig. 13). In case of DOPAMINE oxidation, the velocity calculated from the initial rate has been plotted against pH (Fig. 14). The optimum pH for tyramine oxidation was 7.8. It is interesting to note that at this pH tyramine is oxidised without appreciable lag phase. The pH-activity curve of DOPAMINE showed two optimum pH values at 6.2 and 8.6.

(iii) Effect of Inhibitors - The effect of a number of inhibitors on the oxidation of DOPAMINE by P.somniferum acetone powder extracts is shown in Table XI. At a concentration of 0.005 M semicarbazide, the oxidation of DOPAMINE was strongly inhibite

Fig. 13. *Effect of pH.* The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 100 µmoles of sodium phosphate buffer of varying pH, as indicated, and acetone powder extract equivalent to 520 µg protein. The side arm contained 4 µmoles of tyramine in 0.2 ml.

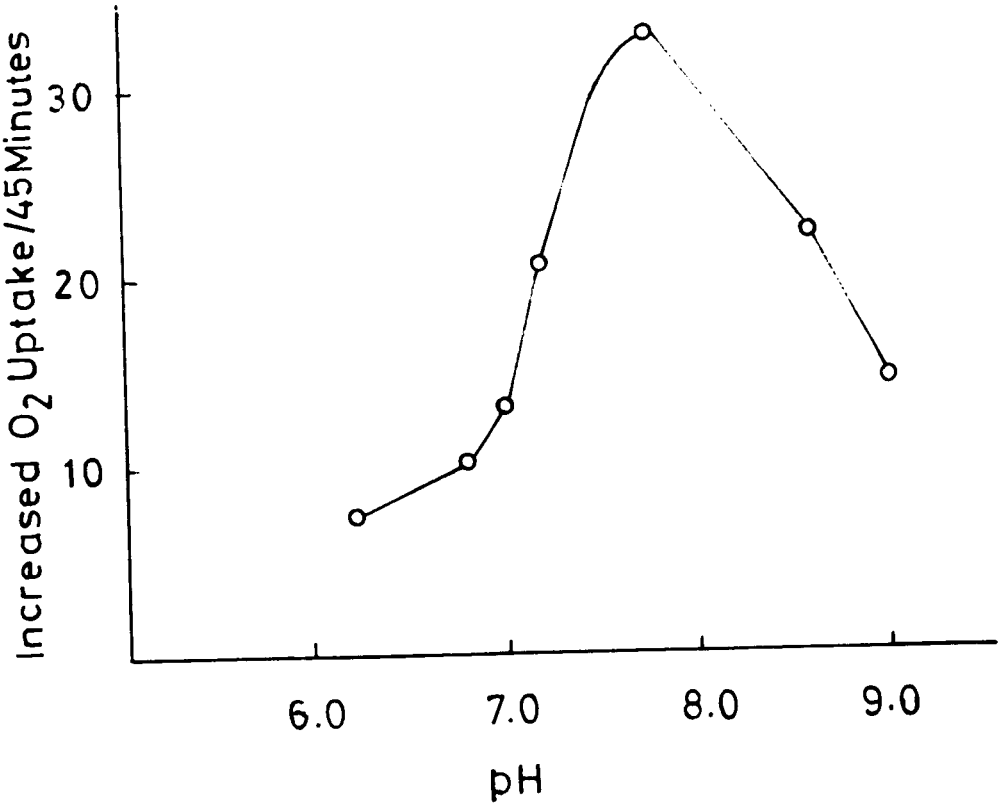

Increased O_2 Uptake/45Minutes
30
20
10
6.0
7.0
8.0
9.0
pH

Fig. 14. Effect of pH. The incubation mixture was the same as described for Fig. 13 except that the substrate used was DOPAMINE.

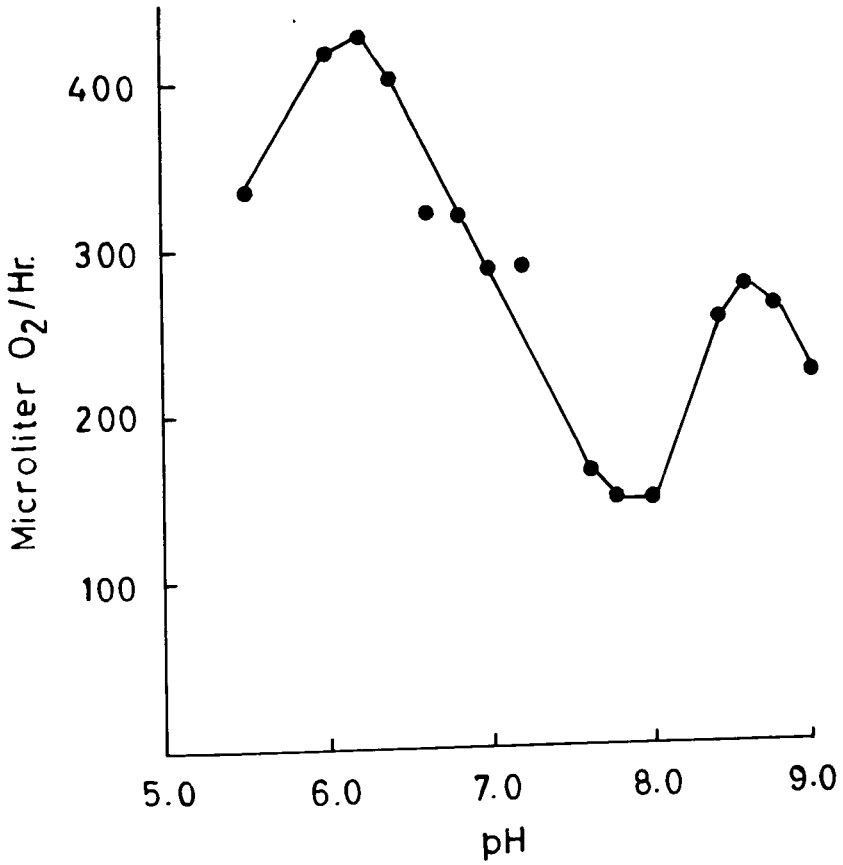

400
300
200
100
Microliter O_2/Hr.
5.0
6.0
7.0
8.0
9.0
pH

Table XI

Effect of Inhibitors

The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 100 µmoles of sodium phosphate buffer, pH 7.0, acetone powder extract equivalent to 520 µg protein and varying concentrations of inhibitors as indicated. The side arm contained 4 µmoles of DOPAMINE in 0.2 ml.

Inhibitor	Concentration	Inhibition
	(µmoles)	(%)
Hydroxylamine	2.0	44
	10	57
	30	57
	40	100
Semicarbazide	1.0	5.0
	2.0	28
	10	52
	30	77
	40	97
Sodium bisulfite	10	87
	20	92
Sodium azide	2.0	17
	10	38
	30	63
	40	68
Thiourea	0.01	67
	0.1	100
Sodium diethyl dithiocarbamate	0.1	35
	2.0	100
Potassium ethyl xanthate	4.0	54
Salicylaldoxime	0.1	52
Isoamylamine	20	86
Cadaverine	20	23
Putrescine	20	32

Hydroxylamine was as effective inhibitor as semicarbazide. Azide at the same concentration was comparatively less effective inhibitor than hydroxylamine and semicarbazide. Sodium bisulfite had a very strong inhibitory effect.

A strong inhibition was observed with sodium diethyl dithiocarbamate, potassium ethyl xanthate, salicylaldoxime and thiourea. These reagents are usually considered as specific inhibitors of copper containing enzymes. The inhibitory effect of thiourea can be considered an additional evidence for the absence of amine oxidase in P.somniferum, since thiourea is known to activate amine oxidase (97).

Certain amines like cadaverine, putrescine and isoamylamine, at higher concentrations were slightly inhibitory to enzyme activity. Isoamylamine was comparatively more strong inhibitor.

(iv) Effect of Metallic Ions - The effect of various metallic ions on the activity of phenolase complex was tested (Table XII). Only those metals which are known to compete with copper were found to have strong inhibitory effect on the activity of enzyme.

Table XII

Effect of Metallic Ions

The incubation mixture was the same as described for Table XI except that varying concentrations of metallic ions were added instead of inhibitors.

Metallic ion	Concentration	Inhibition
	(μmoles)	(%)
Au^{+++}	0.1	11
	0.2	20
	0.5	40
	2.0	83
	5.0	100
Hg^{++}	1.0	3.0
	10	25
	20	46
	40	100
Ag^{+}	4.0	6.0
	10	11
	20	43
	40	100
Cu^{++}	10	9.0
	20	13
Mn^{++}	20	0
	40	15
As^{+++}	5	0
	40	4.0

None of the metals tested was found to activate the enzyme. In contrast to the findings of Bertrand (98), Mn^{++} has no effect on the phenolase complex activity of P.somniferum. The inhibition of phenolase complex by silver, gold and mercury salts further supported the fact that like the enzyme from other sources (73, 74, 99), phenolase complex of P.somniferum is a copper containing protein.

That P.somniferum phenolase complex is indeed a copper containing enzyme was further demonstrated by the following indirect approaches -

(i) The inhibitory effect of sodium diethyl dithiocarbamate is relieved by addition of excess of copper (Table XIII). This type of reactivation of mushroom tyrosinase has been reported by Tenebaum and Jensen (100).

(ii) The inhibitory effect of mercury is relieved by addition of reagents which are known to combine with mercury such as cysteine and glutathione (Table XIV & XV). The addition of these sulfhydryl compounds results in overcoming the inhibition due to mercury which was competing with copper. These

Table XIII

Inhibition of Phenolase Complex by Sodium Diethy Dithiocarbamate and Recovery of its Activity by Added Cupric Ions

The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 100 µmoles of sodium phosphate buffer, pH 7.0, acetone powder extract equivalent to 520 µg protein and 2.0 µmoles of sodium diethyl dithiocarbamate as indicated. The side arm contained 4 µmoles of DOPAMINE in 0.2 ml. Before addition of 2.0 µmoles of copper sulphate, the contents of the flasks were preincubated for 5 minutes. Suitable controls were taken to correct endogeneous uptake of oxygen.

System	Percent activity
Extract + DOPAMINE	100
Extract + DOPAMINE + Sodium diethyl dithiocarbamate	7
Extract + DOPAMINE + Sodium diethyl dithiocarbamate + Cu^{++}	100

Table XIV

Inhibition of Phenolase Complex by Mercuric Ions and Recovery of its Activity by Reduced Glutathione

The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 100 µmoles of sodium phosphate buffer, pH 7.0, acetone powder extract equivalent to 520 µg protein and 40 µmoles of mercuric ions. The contents of the flasks were thoroughly mixed and preincubate for 5 minutes. Different concentrations of reduced glutathione, as indicated, were added. The side arm contained 4 µmoles of DOPAMINE in 0.2 ml. Suitable control flasks were taken to correct for endogeneous uptake of oxygen. The enzyme activity of the flask containing no mercury was taken as 100.

System	Percent Activity
Extract + DOPAMINE	100
Extract + DOPAMINE + Hg^{++}	0
Extract + DOPAMINE + Hg^{++} + glutathione (10 umoles)	9
Extract + DOPAMINE + Hg^{++} + glutathione (20 umoles)	59
Extract + DOPAMINE + Hg^{++} + glutathione (30 umoles)	65
Extract + DOPAMINE + Hg^{++} + glutathione (40 umoles)	68
Extract + DOPAMINE + Hg^{++} + glutathione (50 umoles)	100

Table XV

Inhibition of Phenolase Complex by Mercuric Ions and Recovery Of its Activity by Cysteine

The incubation mixture was the same as described for Table XIV except that varying concentrations of cysteine, as indicated, were added instead of glutathione.

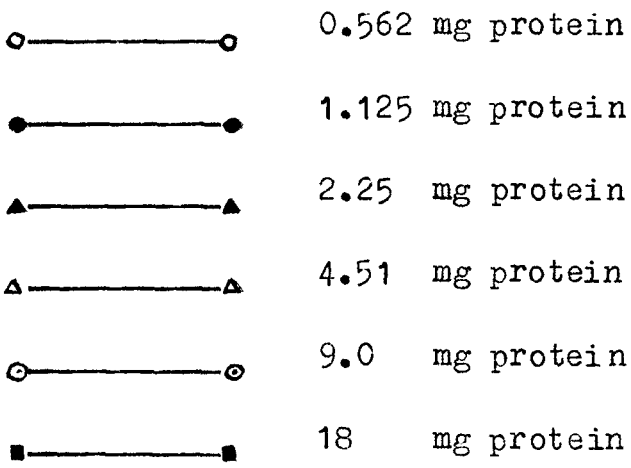
System	Percent Activity
Extract + DOPAMINE	100
Extract + DOPAMINE + Hg^{++}	0
Extract + DOPAMINE + Hg^{++} + cysteine (5 umoles)	4
Extract + DOPAMINE + Hg^{++} + cysteine (10 umoles)	72
Extract + DOPAMINE + Hg^{++} + cysteine (25 umoles)	85
Extract + DOPAMINE + Hg^{++} + cysteine (35 umoles)	85
Extract + DOPAMINE + Hg^{++} + cysteine (50 umoles)	100

sulfhydryl compounds do not activate the enzyme because it has been observed that arsenate or iodoacetate have no appreciable inhibitory effect.

(v) Activity-Enzyme Concentration Relationship - One of the most striking characteristics of P.somniferum phenolase complex is the pronounced inactivation or destruction of the enzyme during the course of reaction. Some typical results are shown in Figs. 15 & 16. Similar results were obtained with 32-fold purified preparations.

It appears that during the oxidation of catechol and DOPAMINE, the rate of oxygen consumption falls off within a short time and unless relatively large amounts of the enzyme are used, the oxidation stops before the substrate is completely oxidised. In other words the oxygen uptake as a function of time as well as enzyme concentration is linear only for a very short time. These observations may be due to rapid inactivation of the enzyme during the course of reaction. Such inactivation

Fig. 15. Effect of enzyme concentration on DOPAMINE oxidation. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 200 µmoles of sodium phosphate buffer, pH 7.0, 50% (w/v) leaf homogenate equivalent to different concentrations of proteins. The side arm contained 30 µmoles of DOPAMINE in 0.2 ml.



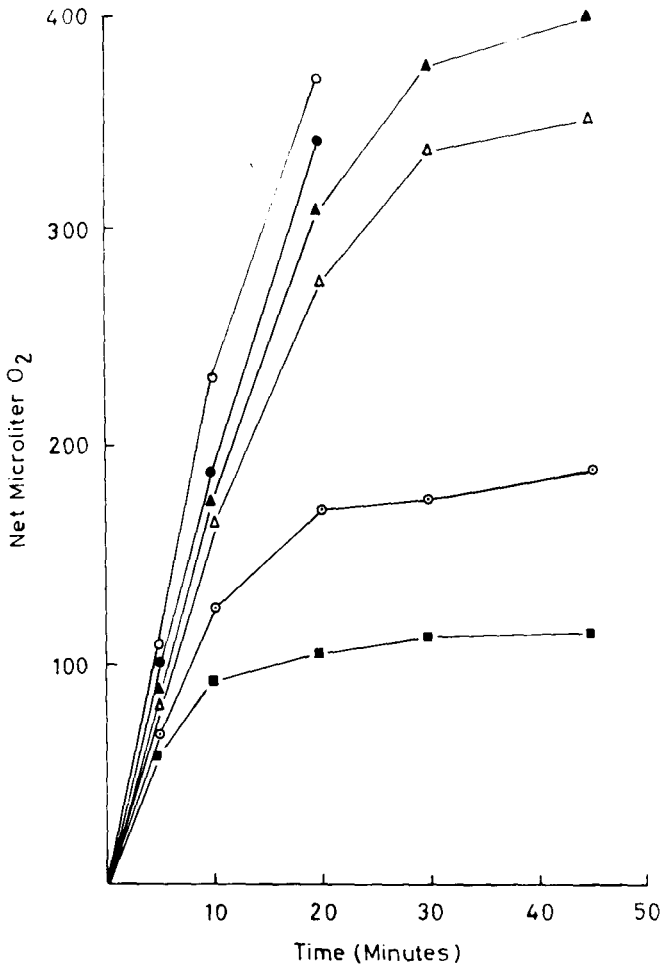
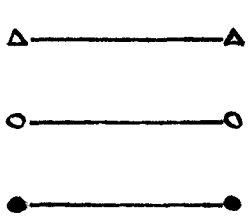
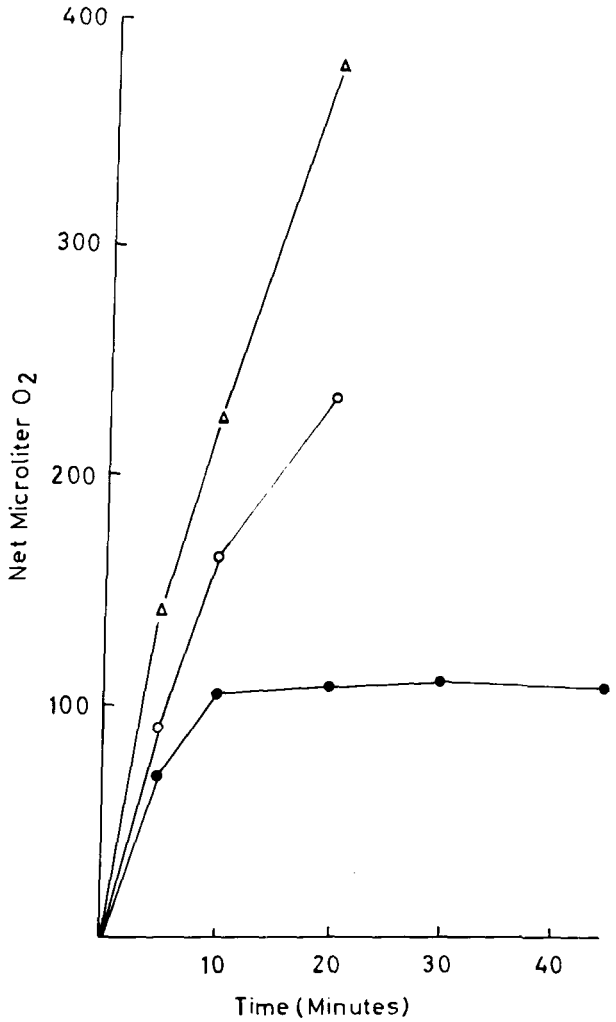

400
300
200
100
Net Microliter O_2
10
20
30
40
50
Time (Minutes)

Fig. 16. Effect of enzyme concentration on catechol oxidation. The incubation mixture was the same as described for Fig. 15 except that substrate used was catechol.



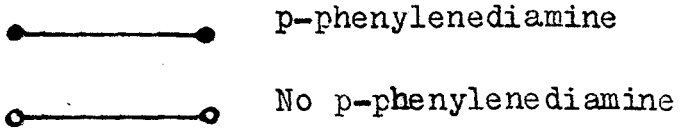

400
300
200
100
Net Microliter O_2
10
20
30
40
Time (Minutes)

characteristics has been reported with phenolase complex of other sources (76, 77, 79, 93, 101, 102).

(vi) Effect of p-phenylenediamine - p-phenylenediamine is known to combine with quinones (103). Therefore, it was envisaged that if product inhibition is responsible for inactivation process observed above, addition of p-phenylenediamine will activate the enzyme by protecting it against inactivation caused by the product of reaction. However, a decrease in the rate of oxygen uptake was observed in presence of p-phenylenediamine which was accompanied by an increase in the duration of linearity (Fig. 17).

(vii) Inhibition of Ascorbic Acid Oxidase - The continuous uptake of oxygen by acetone powder extracts of P.somniferum in presence of ascorbic acid indicated the presence of high ascorbic acid oxidase activity in the system. The oxygen uptake with ascorbic acid and DOPAMINE together was less than the sum of the oxygen uptakes when they are present seperately. These results are presented in Fig. 18. Since almost complete oxidation of

Fig. 17. Effect of p-phenylenediamine. The incubation mixture was the same as described for Table XI except that 20 μmoles of p-phenylenediamine were used instead of inhibitor.



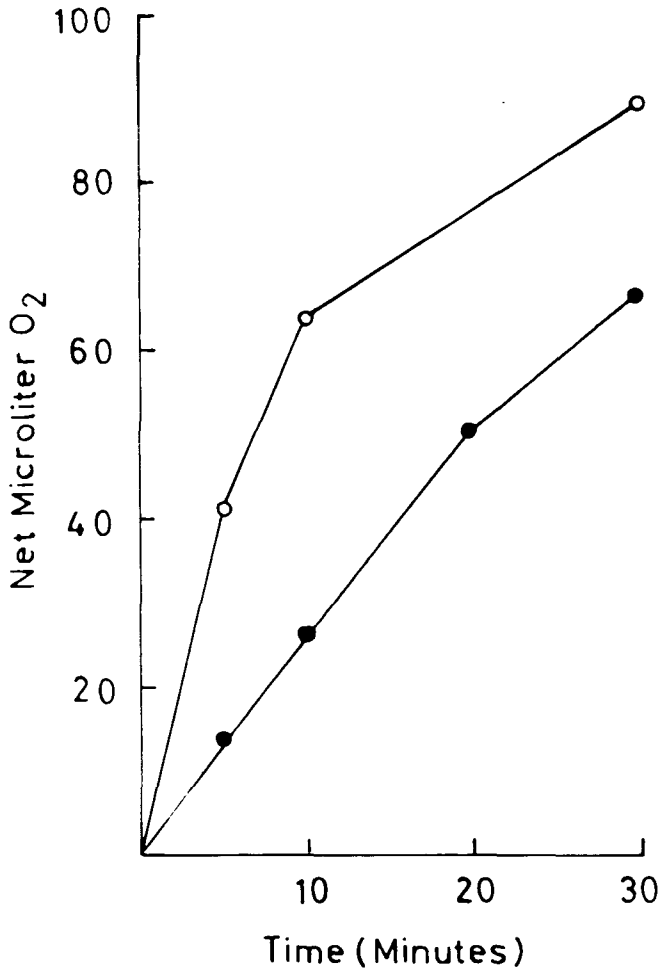
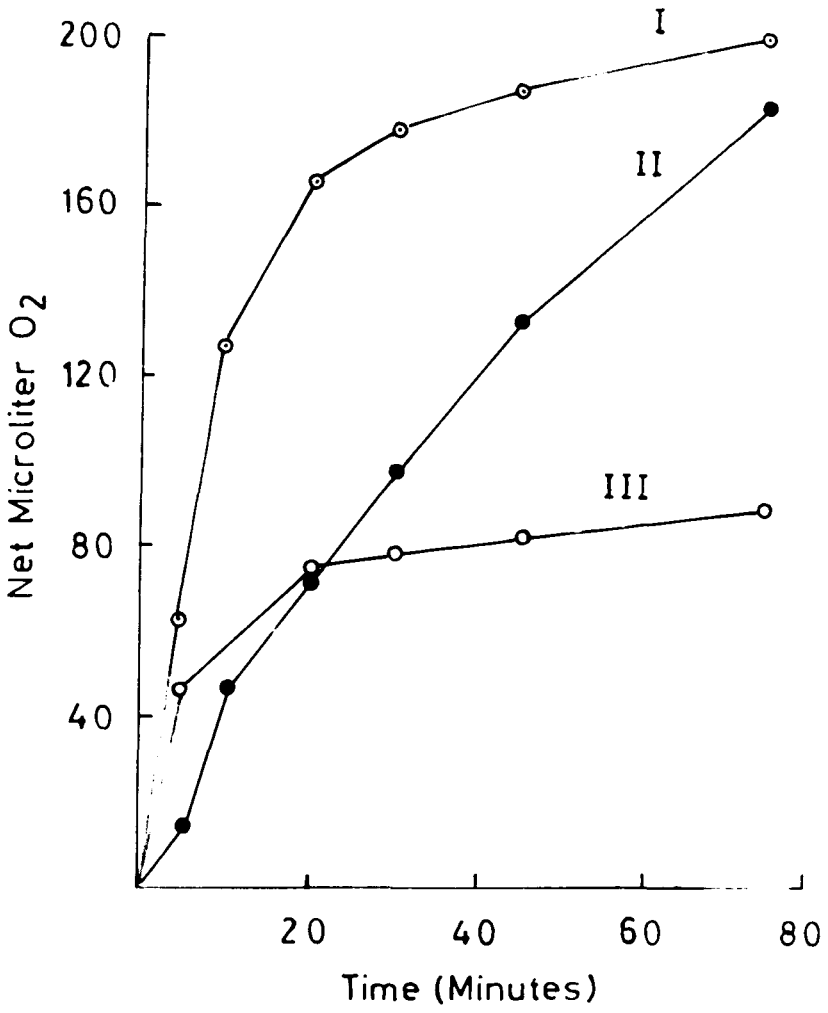

100
80
60
40
20
Net Microliter O_2
10
20
30
Time (Minutes)

Fig. 18. Inhibition of ascorbic acid oxidase by phenolase complex of P.somniferum. The main compartment of Warburg flask, in a total volume of 1.6 ml, contained 100 µmoles of sodium phosphate buffer, pH 7.0, and acetone powder extract equivalent to 520 µg protein. One of the side arm contained 4 µmoles of DOPAMINE in 0.2 ml while the second contained 20 µmoles of ascorbic acid in 0.2 ml. The center well contained 0.2 ml of 20% KOH. After equilibration for 10 minutes the contents of the both side arms were simultaneously tipped into the main compartment of the flask. Suitable controls were included to correct for endogeneous uptake of oxygen.

Curve I. Oxidation of DOPAMINE and ascorbic acid.

Curve II. Oxidation of ascorbic acid.

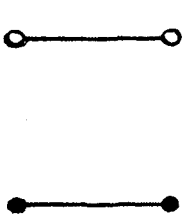
Curve III. Oxidation of DOPAMINE.


200
160
120
80
40
Net Microliter O_2
I
II
III
20
40
60
80
Time (Minutes)

DOPAMINE occurs during the early stages of oxidation (30 to 45 minutes), it was unlikely that DOPAMINE oxidation was inhibited. The decrease in oxygen uptake in presence of ascorbic acid and DOPAMINE may be perhaps due to the inhibition of ascorbic acid oxidase by phenolase complex - DOPAMINE system. It is worthwhile to mention here that inactivation of pepsin, trypsin, chymotrypsin and lysozyme in presence of catechol and inactivation of invertase in absence of catechol by phenolase complex from other sources has already been reported (104, 105, 106,107).

8. Formation of Quinones - Figs. 19 & 20 show the amount of quinones formed due to the oxidation of catechol, DOPAMINE tyramine and p-cresol. The shapes of the curves show that not only the formation of quinone was less rapid but the quinone formation never reached completion and its formation was soon followed by a sharp disappearance, specially when catechol and DOPAMINE were used as substrates. It is not surprising that under the assay conditions there is a sharp drop in the amount of quinones, since the stability of quinones depends upon many factors. It is known from the work of Ball and Chen (108) that

Fig. 19. Formation of quinones. The incubation mixture, in a total volume of 2 ml, contained 100 μmoles of sodium phosphate buffer, pH 7.0, 10 μmoles of substrate, and acetone powder extract equivalent to 520 μg of protein.



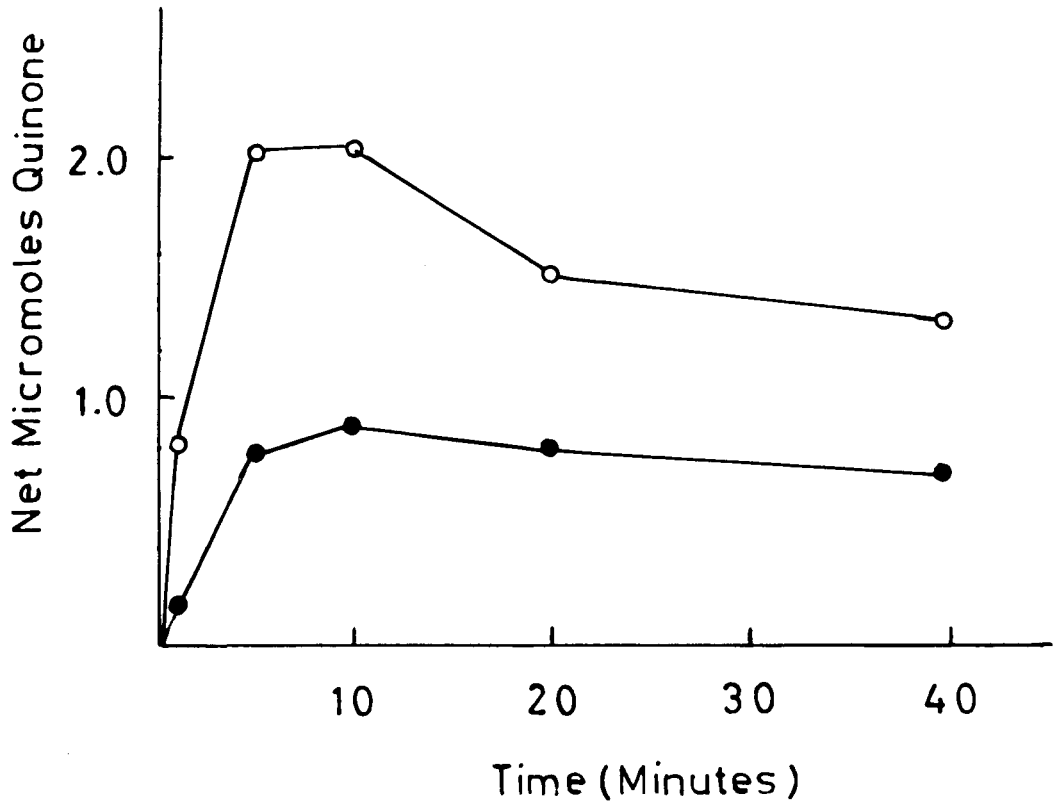
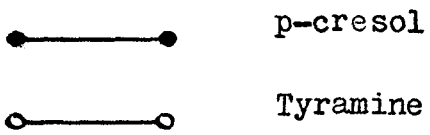
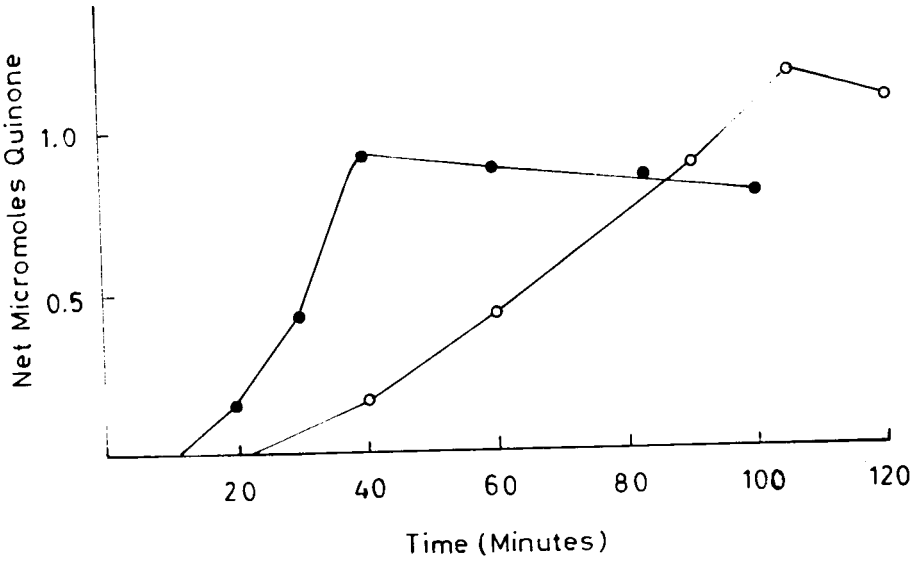

Net Micromoles Quinone
2.0
1.0
10
20
30
40
Time (Minutes)

Fig. 20. Formation of quinones. The incubation mixture was the same as described for Fig. 19 except that tyramine and p-cresol were used as substrate.




Net Micromoles Quinone
1.0
0.5
20
40
60
80
100
120
Time (Minutes)

the stability of quinones diminishes very rapidly as the pH of the aqueous solution is increased beyond pH 6.8. Likewise from the observations made by Dawson and Nelson (109), it is evident that the unoxidised catechol concentration plays a very important role in the disappearance of quinone and that this effect of unoxidised catechol is also dependent on pH. The same authors have also demonstrated that at lower pH values the disappearance of quinones in dilute solutions is a first order reaction but as the initial concentration of the quinone is increased, the rate of disappearance becomes too rapid to be accounted for.

9. Isolation of Quinones - The quinones formed by the action of P.somniferum phenolase complex on p-cresol and catecol were trapped with aniline. A preliminary control experiment showed that aniline is neither oxidised by P.somniferum acetone powder extracts nor inhibits the phenolase complex activity. Although DOPA and DOPAMINE formed quinones, attempts to isolate them by trapping with aniline failed. This is in agreement with the findings of Pough and Raper (62) who also showed that although

DOPA gives rise to quinone, its trapping as anilino derivative was not possible.

(i) Isolation of Dianilino-o-benzoquinone - The method was essentially the same as used by Pough and Raper (62).

2 gms. of catechol and 4 ml of freshly distilled aniline were dissolved in about 250 ml of 0.1 M sodium phosphate buffer, pH 7.0. To this was added 250 ml of 20% acetone powder extract. The incubation mixture was shaken at 28° for four hours. In a few minutes a red colour developed, the liquid clouded and after two days red crystalline needles seperated. Finally, incubation mixture was extracted with ether in a liquid-liquid extractor. The etheral layer was extracted with 0.02 N NaOH. The NaOH layer was acidified with 2% HCl and the red precipitate filtered, washed with 1% HCl to remove any traces of aniline and crystallized from acetone. The product was recrystallized from acetone and petroleum ether (40 - 60°).

The dianilino-o-benzoquinone was obtained in the form of bright red needles. The crystalline material was soluble in

organic solvents. It dissolved in conc. sulfuric acid giving olive green colour. It was soluble in alkali and alkali carbonates giving a purplish red solution, reprecipitable by dilute acids. Therefore, the isolated compound was identical in all respects to that obtained by Pough and Raper using mealworm enzyme.

Analysis. Calculated for

$C_{18}H_{14}N_2O_2$: C 74.48%; H 4.83%; N 9.66% O 11.03

Found: C 74.10%; H 5.09%; N 9.87% O 10.94

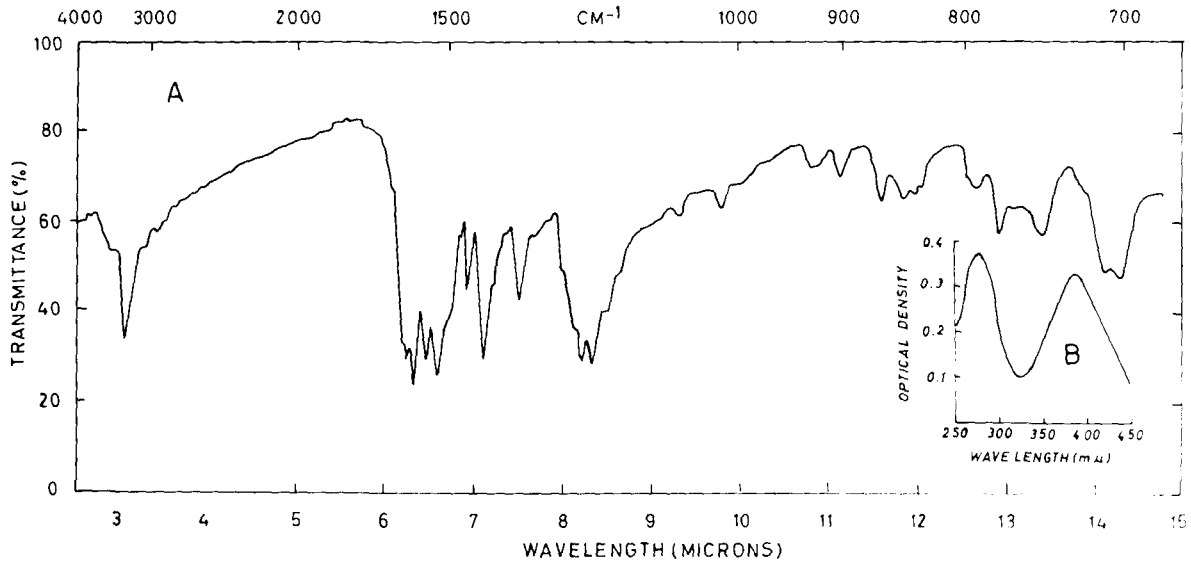
Melting point (uncorrected) reported 193°

Melting point (uncorrected) observed 193°

The I.R. and U.V. spectra of dianilino-o-benzoquinone in are shown in Fig. 20 A and 20 B. The I.R. spectrum of the compound shows $-\overset{|}{N}-H-$ streching absorption at 3250 cm^{-1}, which is indicative of the presence of some hydrogen bonding. The 1450 cm^{-1} to 1650 cm^{-1} region of the spectrum shows several bands which must be assigned to the aromatic nucleus, the quinone carbonyls and the $-\overset{|}{C}=\overset{|}{C}-$ streching of the ortho quinone system. The carbonyl streching in quinone is generally found between 1640 cm^{-1} and

ig. 20 A. I.R. spectrum of dianilino-o-benzoquinone (in KBr).

B. U.V. spectrum of dianilino-o-benzoquinone.


4000
3000
2000
1500
CM-1
1000
900
800
700
100
80
60
40
20
0
A
TRANSMITTANCE (%)
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)
0.4
0.3
0.2
0.1
OPTICAL DENSITY
B
250
300
350
400
450
WAVE LENGTH (mμ)

1675 cm^{-1} which may be occasionally split. In the present case conjugation with the aniline moiety should lead to a further bathochrome shift in the >C=O streching. The compound can, therefore, be regarded both as a para substituted quinone and as a vinylogous secondary amide and these structural features might account for the extremely low frequency of the >C=O streching band which occurs, in this case, at 1585 shoulder - 1572 cm^{-1}. The -N-H- bending in secondary amides occurs usually at 1550 cm^{-1} The 1542 cm^{-1} and 1512 cm^{-1} bands can therefore be assigned to th -N-H- bending of secondary amide and might also contain the aromatic bands in the region. The 1610 cm^{-1} and 1599 cm^{-1} bands can be assigned to -C=C- of the quinone and benzene rings. It is, however, difficult in the presence of corroborative data to certain of the assignments in this region. A mono substituted aromatic nucleus is quite clearly shown by four bands between 690 cm^{-1} to 775 cm^{-1}.

As shown, the U.V. absorption maxima of the compound is a 390 m μ which is characteristic of o-benzoquinone system.

(ii) Isolation of Dianilinohomoquinoneanil - The method was essentially the same as described by Pough and Raper (62).

2 gms. of p-cresol and 5 ml of freshly distilled aniline were dissolved in about 250 ml 0.1 M sodium phosphate buffer, pH 7.0, and 250 ml of 20% acetone powder extract was added. The liquid became reddish and then a reddish brown precipitate appeared after two days. The reaction mixture was extracted with ether in liquid-liquid extractor. The ether layer was then evaporated. The product thus obtained was crystallized from aceton

The brownish red needles obtained were insoluble in alkali but soluble in organic solvents. With conc. sulfuric acid it gave a brownish crimson colour, becoming brown then yellow on dilution. The isolated compound was, therefore, identical in all respects to that of dianilinohomoquinoneanil (62).

Analysis. Calculated for

$C_{25} H_{21} N_3 O$: C 79.15%; H 5.54%; N 11.08%; O 4.23

Found: C 78.93%; H 5.37%; N 11.11%; O 4.59

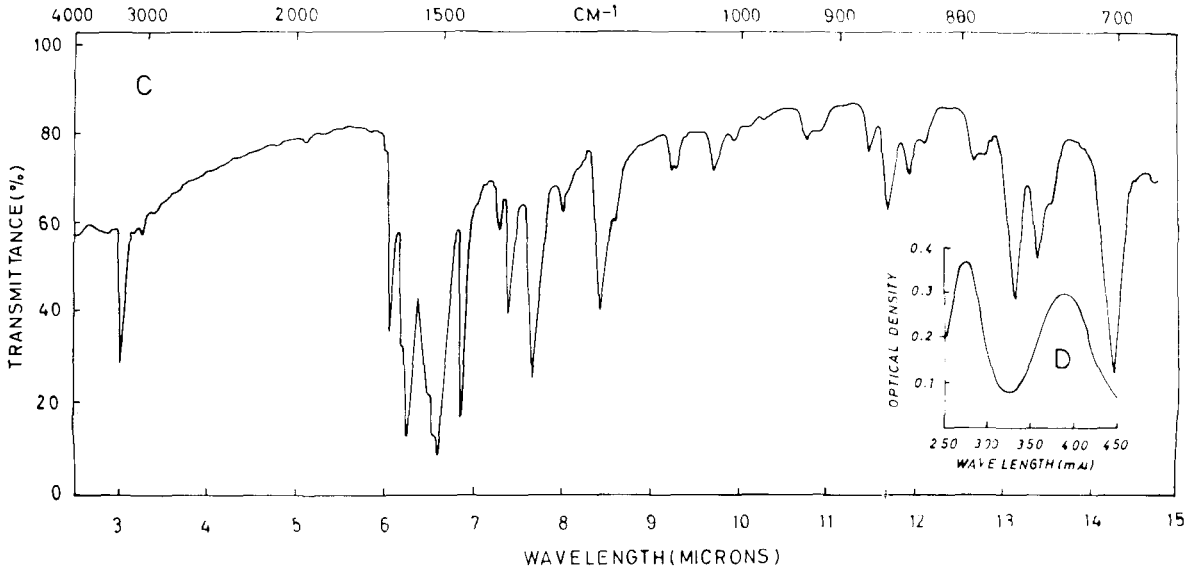
Melting point (uncorrected) reported 202°

Melting point (uncorrected) observed 201°

The I.R. and U.V. spectra of dianilinohomoquinoneanil are shown in Figs. 20 C and 20 D. The I.R. spectrum of the compound is similar

Fig. 20 C. I.R. spectrum of dianilinohomoquinoneanil (in KBr).

D. U.V. spectrum of dianilinohomoquinoneanil.


4000
3000
2000
1500
CM-1
1000
900
800
700
100
80
60
40
20
0
C
TRANSMITTANCE(%)
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH(MICRONS)
0.4
0.3
0.2
0.1
OPTICAL DENSITY
D
250
300
350
400
450
WAVE LENGTH(mμ)

to that of dianilino-o-benzoquinone but the absorption pattern in the 1500 cm^{-1}-1600 cm^{-1} region is simplified and shows bands at 1632 cm^{-1} (>C=O), 1600 cm^{-1}, 1578 cm^{-1} (aromatic) and a very broad prominant band at 1500 cm^{-1} (-N-H-bending and aromatic). The aromatic nucleus is again characterized by four bands between 690 cm^{-1} to 775 cm^{-1}.

The U.V. absorption maxima of the compound is again at 390 m μ similar to dianilino - o - benzoquinone.

10. Purification of Phenolase Complex of P.somniferum - All manuplations were carried out at 5° unless mentioned otherwise.

(i) Fractional Precipitation with Ammonium Sulfate - 10% (w/v) acetone powder extract of whole plant was prepared in 0.01 M sodium phosphate buffer, pH 7.0, as described earlier. To this was added solid ammonium sulfate to make it 60% saturated. It

was kept for one hour with occasional stirring and then centrifuged at 10,000 X g for 15 minutes. The bulk of the phenolase complex present in acetone powder extract was precipitated at this stage. The ammonium sulfate precipitate was dissolved in 0.01 M sodium phosphate buffer, pH 7.0, dialyzed against two changes of 40 volumes 0.01 M phosphate buffer, pH 7.0, for 18 hours (Dialyzed Ammonium Sulfate Precipitate).

(ii) Fractionation of Enzyme on CM-cellulose Column - The Dialyzed Ammonium Sulfate Precipitate solution was passed through the column at a rate of 1 ml / 5 minutes. The enzyme was purified by 'starting condition' procedure. The conditions were chosen such that the enzyme was separated by elution with equilibrating buffer. The column was eluted with 60 ml of 0.01 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0. The eluate was collected in 10 ml fractions. The enzyme assays were made on every fraction after appropriate dilution. The enzyme was not absorbed on the column and the bulk of the activity appeared in first two fractions.

(iii) Fractionation of Enzyme on DEAE - cellulose Column - The combined active fractions of enzyme obtained by fractionation on CM-cellulose column were passed through DEAE-cellulose column at a flow rate of 1 ml / 5 minutes. Elution was started with 0.2 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0, and 5 ml fractions were collected. The concentration of sodium chloride was carried upto 0.4 M. Enzyme assays were made on every fraction after appropriate dilution.

The summary of the yeilds and degree of purification of a typical preparation of phenolase complex is shown in Table XVI. The specific activity of acetone powder extract was 200. This was increased to about 300 by 60% ammonium sulfate treatment. The ammonium sulfate precipitate solution showed no loss of activity during dialysis against distilled water or 0.01 M sodium phosphate buffer, pH 7.0, even when this was prolonged for several days. During prolonged dialysis against distilled water the enzyme tended to precipitate but the precipitate could be redissolved on addition of phosphate buffer. The dialyzed enzyme when passed on CM-cellulose column was left unabsorbed and bulk of

Table XVI

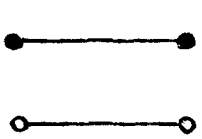
Purification of Phenolase Complex of P.somniferum

The enzyme assay was done manometrically. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 80 µmoles of sodium phosphate buffer, pH 7.0, and requisite amount of protein. The side arm contained 4 µmoles of DOPAMINE in 0.2 ml. The center well contained 0.2 ml of 20% KOH. Under the conditions of assay, one unit of phenolase complex is defined as the amount of enzyme responsible for the uptake of one microliter oxygen per hour. The specific activity has been expressed in terms of enzyme units per mg protein.

Proceedure	Volume	Total units	Total Protein	Specific activity	Purification	Yeild
	(ml)		(mg)			
Acetone Powder Extract	43	28853	144	200	1	100
Dialyzed Ammonium Sulfate Precipitate	14	28391	97	292	1.5	99
CM-cellulose	20	25406	15.2	1400	7	83
DEAE-cellulose	25	15352	2.36	6498	32	54
DEAE-cellulose (Fraction Nos. 8 & 9)	10	10700	1.13	9415	47	37

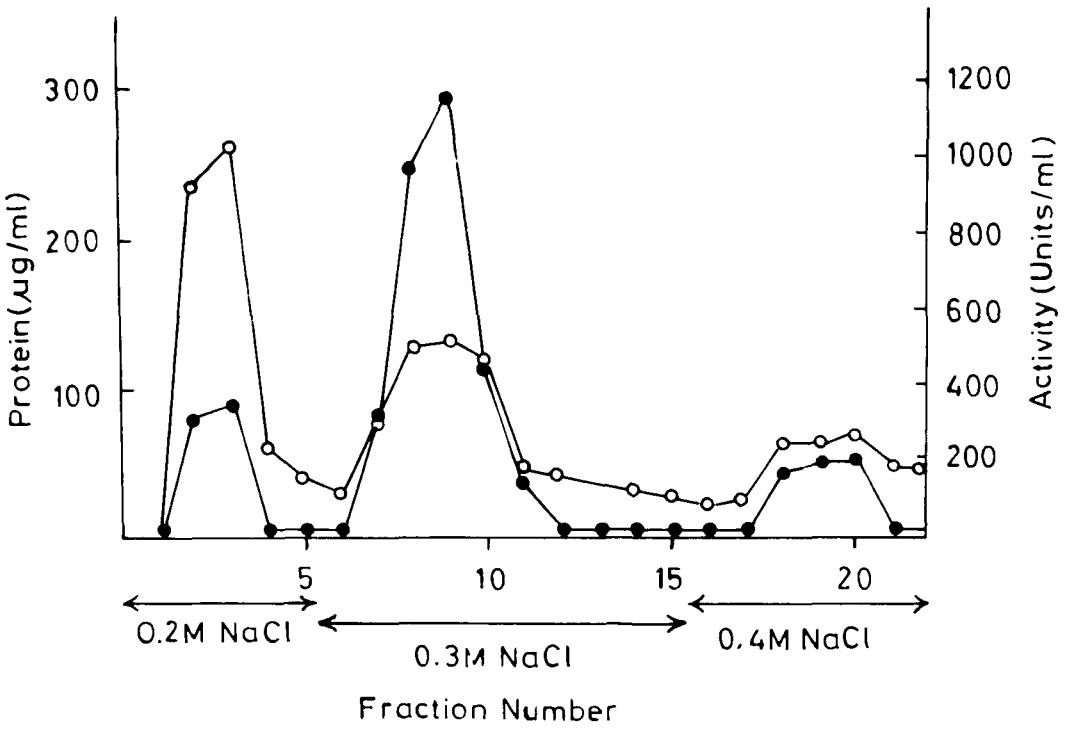
the activity was obtained in first two fractions with an average specific activity value of 1400. Fig. 21 shows the fractionation of active combined fractions obtained by CM-cellulose chromatography on DEAE-cellulose column. Determinations of the proteins of the fractions suggested that under the given conditions proteins were partially seperated into three main parts. All those fractions which were found to contain phenolase complex activity were yellow. The fractions in peak B (7-11) which contained bulk of the enzyme were yellowest. The fractions on either side of these peaks were colourless although they contained protein. The existance of the two minor peaks A and C may be due to the artifact of the method. The average specific activity of the pooled fractions of main peak B was 6498 showing a 32-fold purification over the acetone powder extract. However, the average specific activity of fraction Nos. 8 and 9 was 9415 showing a 47-fold purification over the initial activity. The preparations were stable at - 10° for three weeks. However, complete loss of activity was observed after three months.

Fig. 21. Elution profile of phenolase complex from DEAE-cellulose column (1 X 15 cm.)



Phenolase complex

Protein

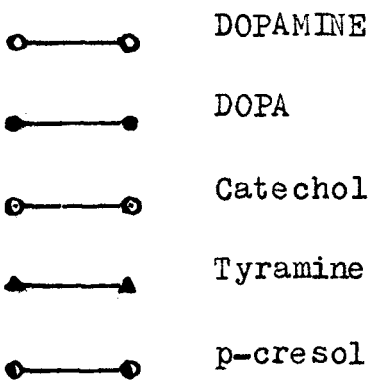

300
200
100
Protein(μg/ml)
1200
1000
800
600
400
200
Activity (Units/ml)
5
10
15
20
0.2M NaCl
0.3M NaCl
0.4M NaCl
Fraction Number

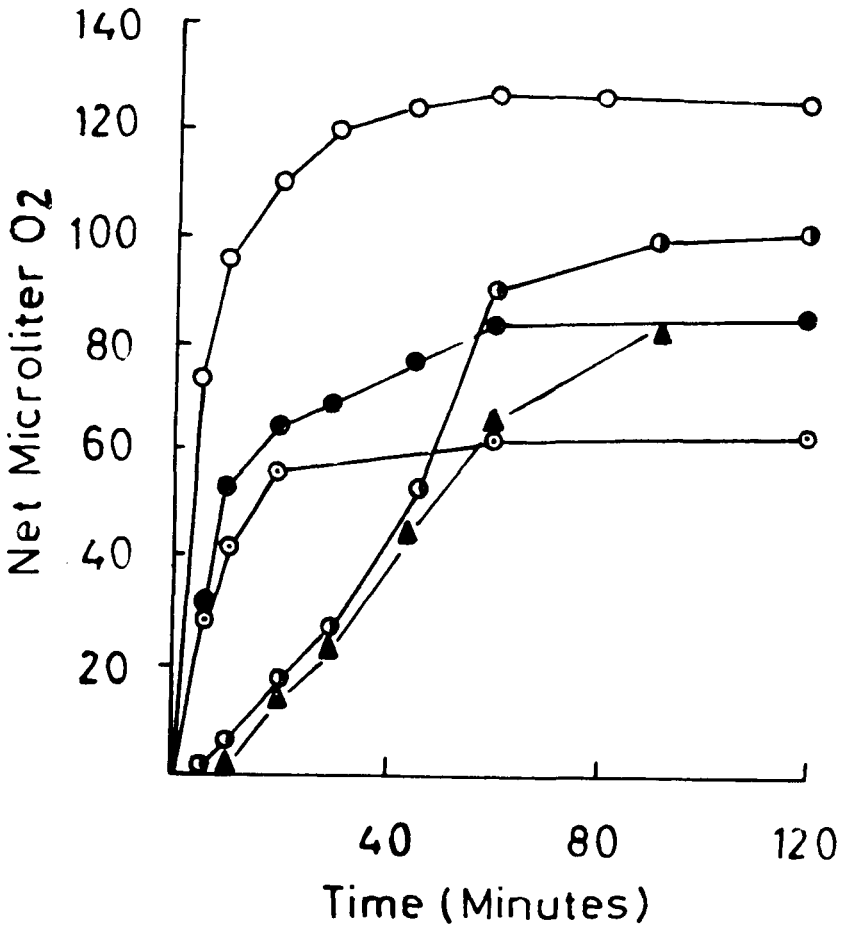
11. Studies on Partially Purified Phenolase Complex of P.somniferum -

(a) Substrate Specificity - Using a substrate concentration of 0.002 M, the catalytic activity of phenolase complex preparations towards a number of different substrates was tested manometrically at pH 7.0. All the substrates, DOPA, DOPAMINE, tyramine, p-cresol and catechol, which were oxidised by crude preparations, were also oxidised by purified ones (Fig. 22). A slight difference was observed in the ratio of oxidation of diphenols to that of monophenols as compared with crude preparations. This may be attributed to the shortening of the lag phase of monophenol oxidation after purification and not due to the presence of two enzymes monophenolase or diphenolase as it is well recognised that single enzyme phenolase complex catalyses the oxidation of mono - as well as di-phenols. None of the phenolic substances, like tyrosine, vanillin or hydroquinone, not oxidised by crude preparations could be oxidised by purified preparations.

(b) Effect of pH - The variation of activity with pH was tested in sodium phosphate buffers with 0.002 M concentration of

Fig. 22. Oxidation of various phenolic substrates by partially purified phenolase complex preparations of P.somniferum. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 80 µmoles of sodium phosphate buffer, pH 7.0, and 94 µg of 32 fold purified enzyme protein. The side arm contained 4 µmoles of substrate, as indicated, in 0.2 ml.



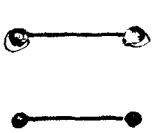

140
120
100
80
60
40
20
Net Microliter O2
40
80
120
Time (Minutes)

different substrates. There was no appreciable difference in pH of the incubation mixture before and after the reaction. The pH - activity curves of monophenols, e.g., p-cresol and tyramine show optima at pH 7.8 (Fig. 23). The same observations were recorded with crude preparations. The diphenols on the other hand exhibited the lack of a well defined optimum pH. The two optimum pH vaules which were noted with crude preparations for DOPAMINE could not be demonstrated with purified preparations. However, maximum oxygen uptake was observed within pH range of 6.5 to 8.5 as shown in Fig. 24.

(c) Effect of Substrate Concentration - The effect of substrate concentration on the rate of oxidation of p-cresol, tyramine, DOPA, DOPAMINE and catechol in phosphate buffer at pH 7.0 is shown in Figs. 25, 26, 27, 28 and 29. As shown in Fig. 28, higher concentrations of tyramine are inhibitory. It is apparent from Km values summarized in Table XVII that DOPAMINE and DOPA are more natural substrates for the phenolase complex of P.somniferum.

(d) Effect of Inhibitors - Partially purified preparations of

Fig. 23. Effect of pH. The incubation mixture was same as described for Fig. 22 except that pH of sodium phosphate buffer varied as indicated.



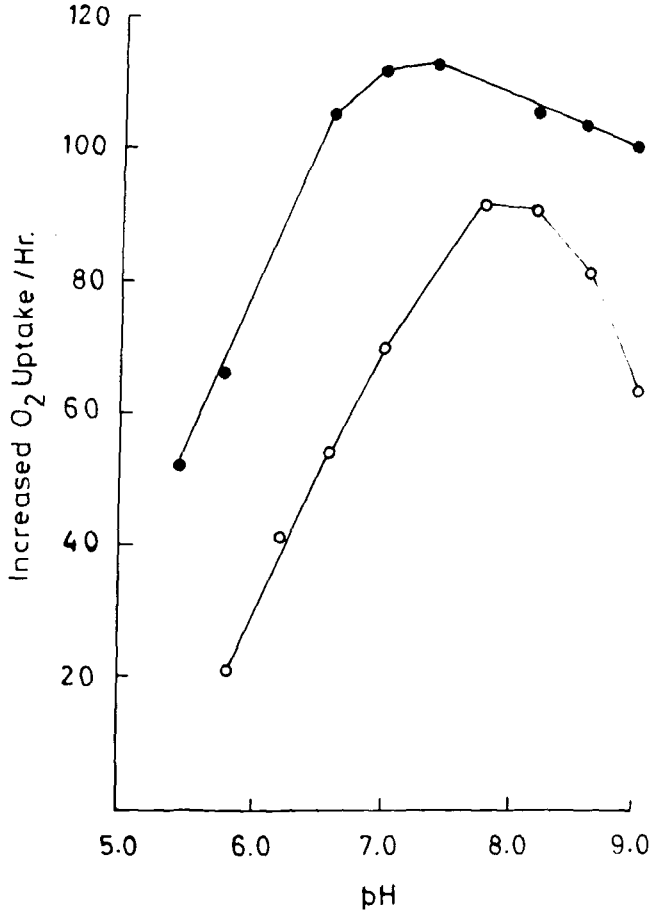
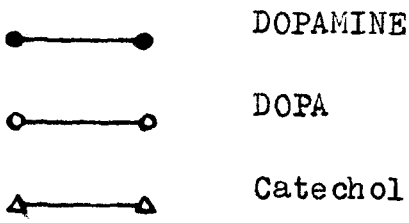

120
100
80
60
40
20
Increased O_2 Uptake / Hr.
5.0
6.0
7.0
8.0
9.0
pH

Fig. 24. Effect of pH. The incubation mixture was the same as described for Fig. 22 except that the pH of the sodium phosphate buffer varied as indicated.



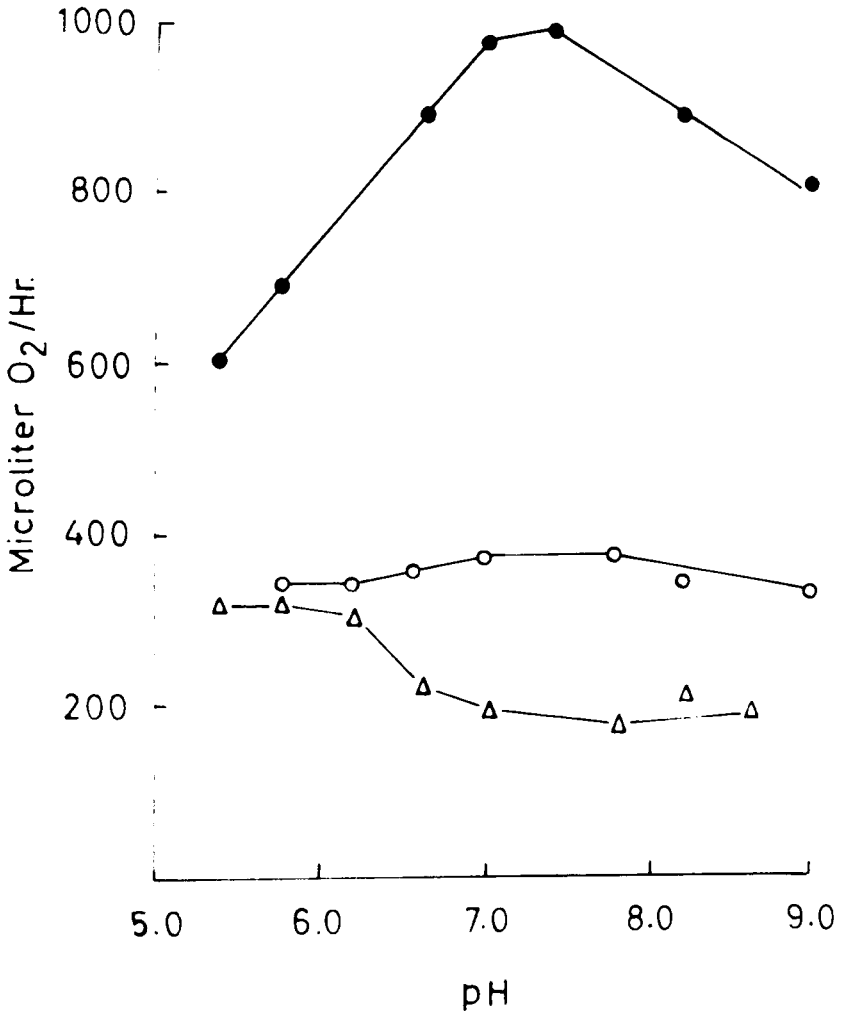

1000
800
600
400
200
Microliter O_2/Hr.
5.0
6.0
7.0
8.0
9.0
pH

Fig. 25. Effect of DOPAMINE concentration on oxygen uptake. The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 80 μmoles of sodium phosphate buffer, pH 7.0, and 94 μg of 32 fold purified enzyme protein. The side arm contained varying concentrations of DOPAMINE, as indicated, in 0.2 ml.

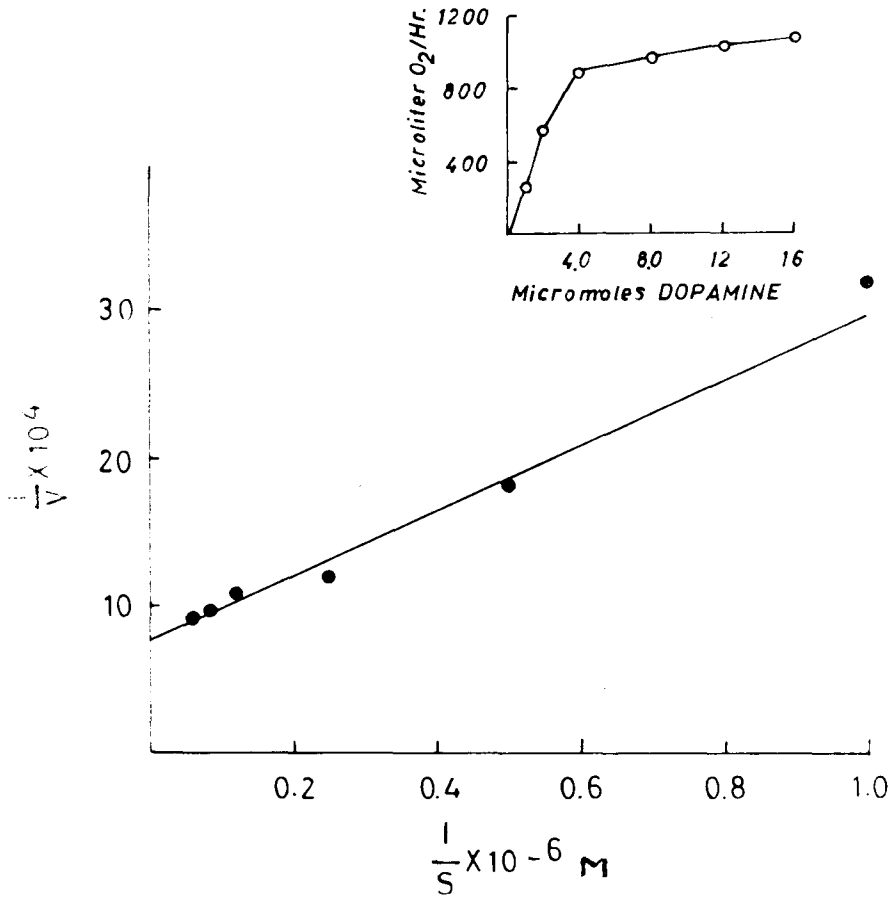

Microliter O_2/Hr.
1200
800
400
4.0
8.0
12
16
Micromoles DOPAMINE
30
20
10
$\frac{1}{V} \times 10^4$
0.2
0.4
0.6
0.8
1.0
$\frac{1}{S} \times 10^{-6}$ M

Fig. 26. Effect of DOPA concentration on oxygen uptake. The incubation mixture was the same as described for Fig. 25 except that DOPA was used as substrate.

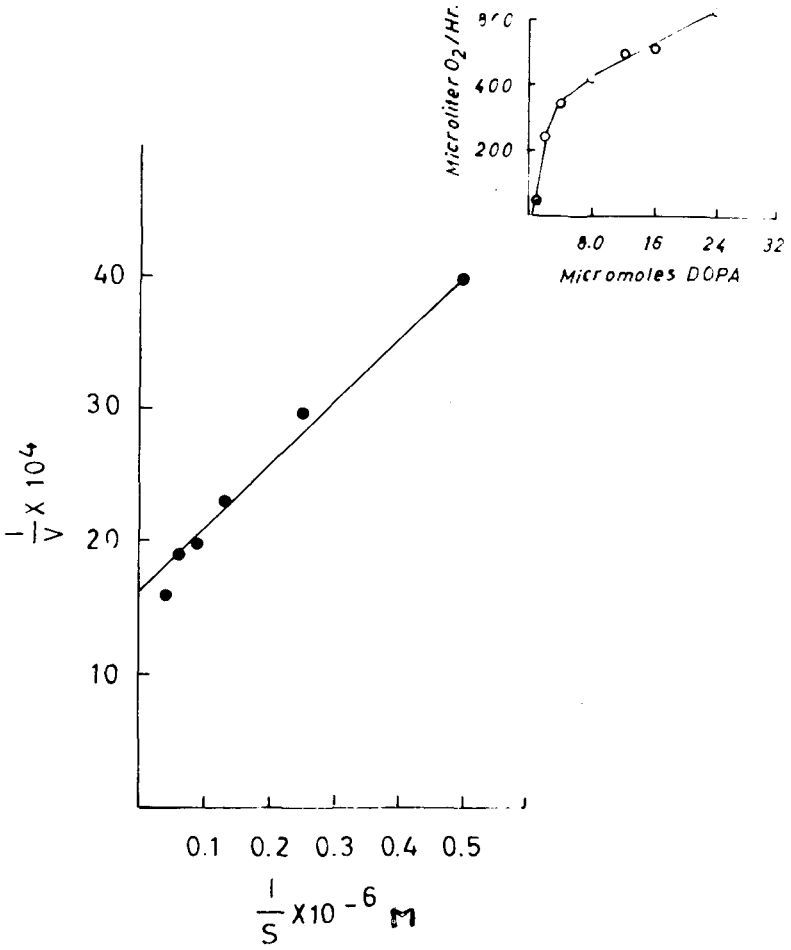

Microliter O_2/Hr.
600
400
200
8.0
16
24
32
Micromoles DOPA
40
30
20
10
$\frac{1}{V} \times 10^4$
0.1
0.2
0.3
0.4
0.5
$\frac{1}{S} \times 10^{-6}$ M

Fig. 27. Effect of catechol concentration on oxygen uptake. The incubation mixture was the same as described for Fig. 25 except that substrate used was catechol.

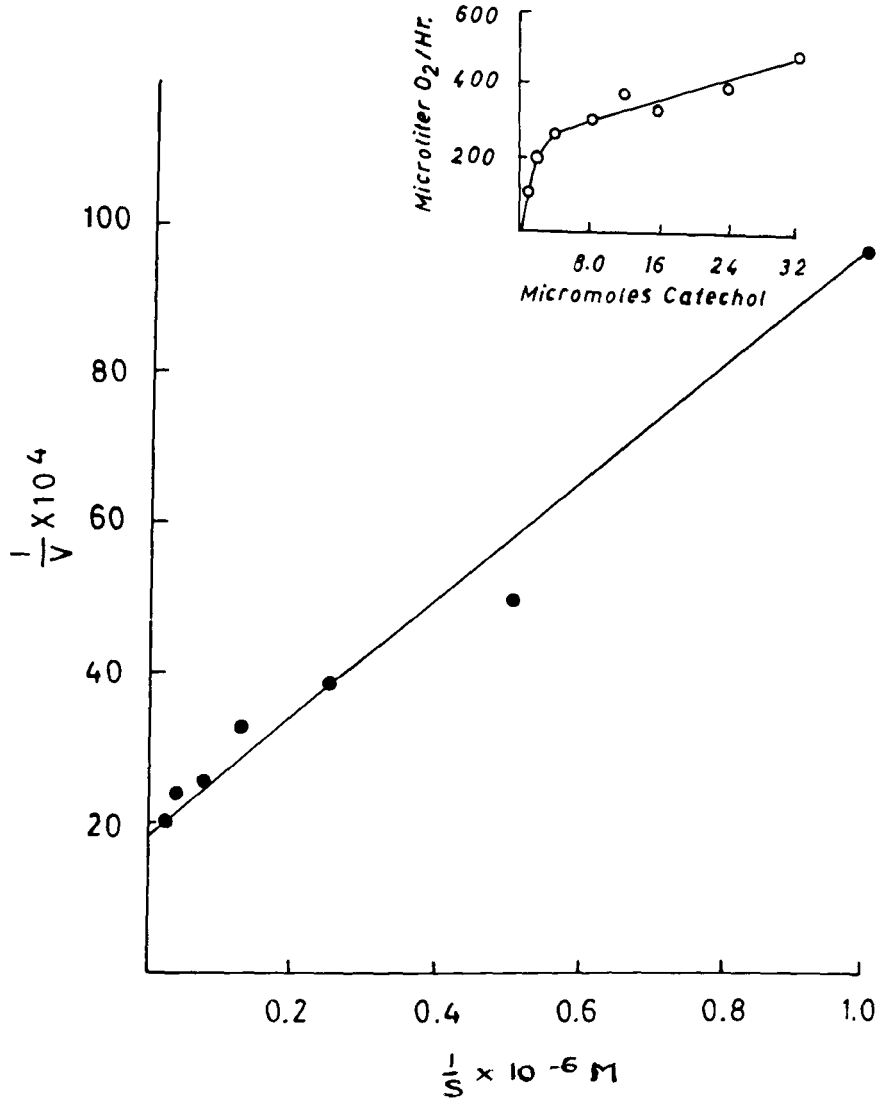

Microliter O_2/Hr.
600
400
200
8.0
16
24
32
Micromoles Catechol
100
80
60
40
20
$\frac{1}{V} \times 10^4$
0.2
0.4
0.6
0.8
1.0
$\frac{1}{S} \times 10^{-6}$ M

Fig. 28. Effect of tyramine concentration on oxygen uptake. The incubation mixture was the same as described for Fig. 25 except that substrate used was tyramine.

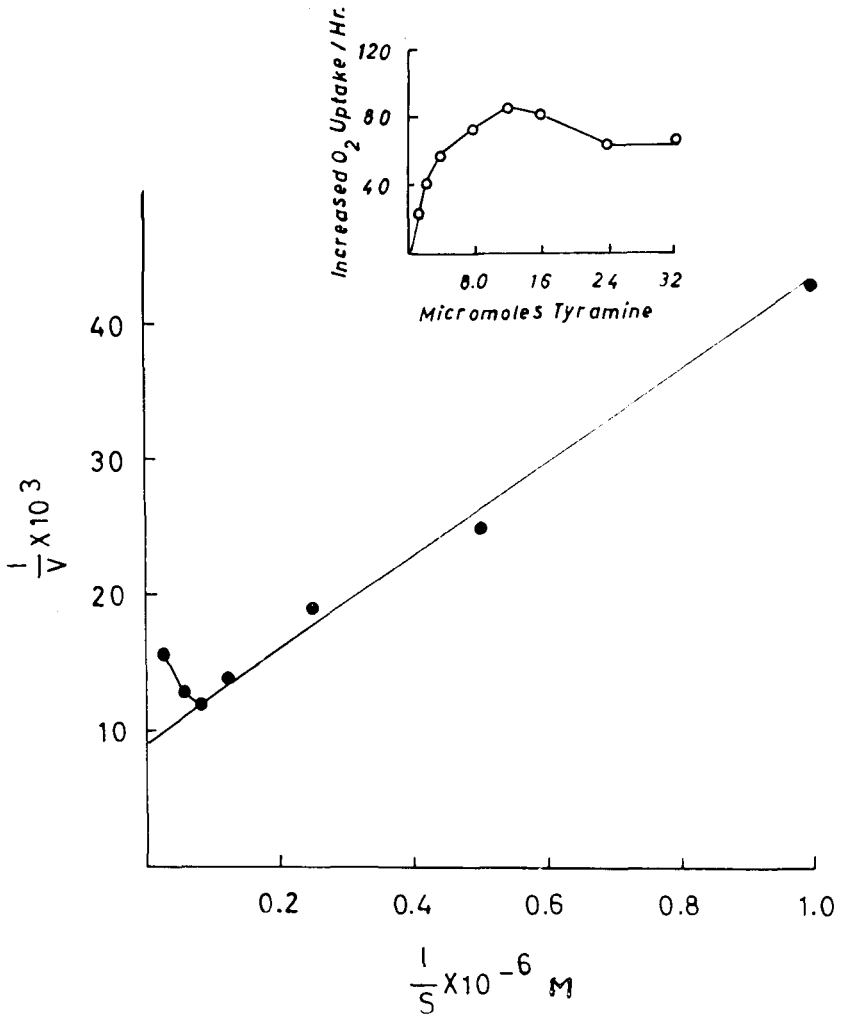

Increased O_2 Uptake / Hr.
120
80
40
8.0
16
24
32
Micromoles Tyramine
$\frac{1}{V} \times 10^3$
40
30
20
10
0.2
0.4
0.6
0.8
1.0
$\frac{1}{S} \times 10^{-6}$ M

Fig. 29. Effect of p-cresol concentration on oxygen uptake. The incubation mixture was the same as described for Fig. 25 except that the substrate used was p-cresol.

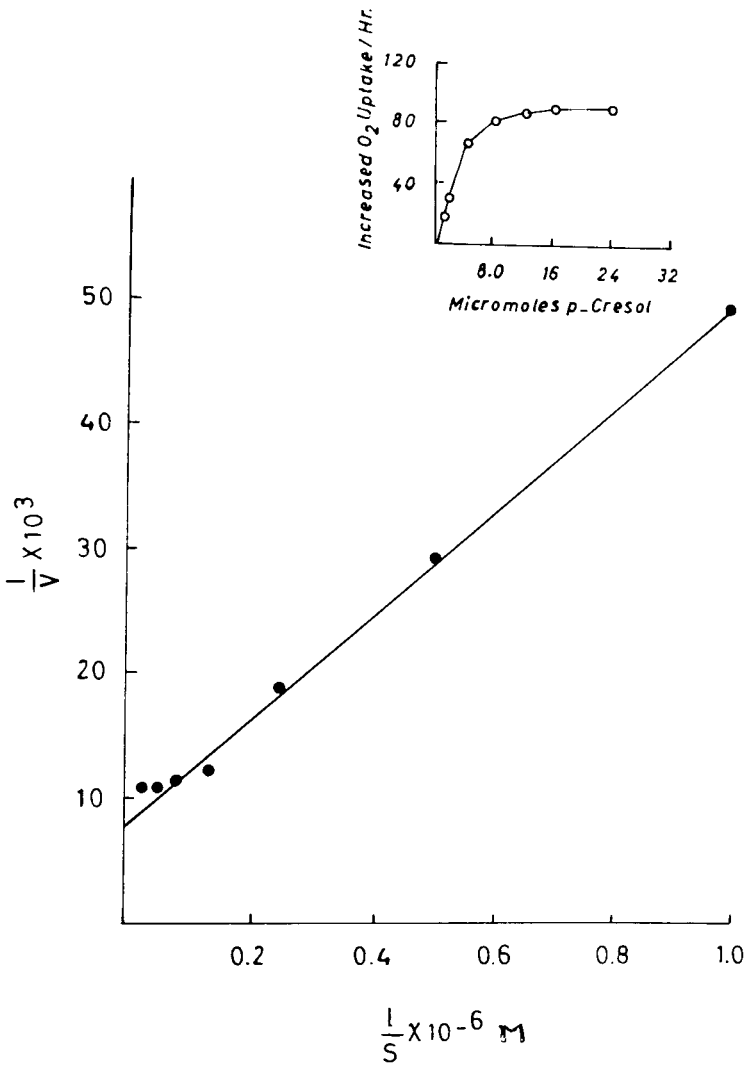

Increased O_2 Uptake / Hr.
120
80
40
8.0
16
24
32
Micromoles p-Cresol
50
40
30
20
10
$\frac{1}{V} \times 10^3$
0.2
0.4
0.6
0.8
1.0
$\frac{1}{S} \times 10^{-6}$ M

Table XVII

Affinity of Various Substrates Towards Phenolase Complex of P.somniferum

Substrate	Km
DOPAMINE	2.5×10^{-7} M
DOPA	3.3×10^{-6} M
Catechol	4.3×10^{-6} M
Tyramine	4.3×10^{-6} M
p-cresol	4.7×10^{-6} M

Table XVIII

Effect of Inhibitors

The main compartment of Warburg flask, in a total volume of 1.8 ml, contained 80 µmoles of sodium phosphate buffer, pH 7.0, 94 µg of 32 fold purified enzyme protein and varying concentrations of inhibitors as indicated. The side arm contained 4 µmoles of DOPAMINE in 0.2 ml.

Inhibitor	Concentration (µmoles)	Inhibition (%)
Potassium cyanide	0.1	37
	0.5	49
	1.0	56
	4.0	100
8-hydroxyquinoline	0.2	12
	0.5	37
	1.0	69
	2.0	100
Sodium diethyl dithiocarbamate	0.1	15
	0.5	91
	1.0	100
Thiourea	0.0025	12
	0.005	16
	0.01	33
	0.05	57
	0.2	100
Semicarbazide	1.0	11
	4.0	47
	20	100

P.somniferum phenolase complex were inhibited by respiratory inhibitors such as cyanide and 8-hydroxyquinoline : 8-hydroxy-quinoline was more strong inhibitor. Semicarbazide was less inhibitory. Sodium diethyl dithiocarbamate and thiourea, specific inhibitors of copper containing enzymes, very strongly inhibited the enzyme activity (Table XVIII).

VII. DISCUSSION

The demonstration of the presence of phenolase complex in P.somniferum is an interesting finding. The enzyme catalyzes the oxidation of p-cresol, tyramine, DOPA, DOPAMINE and catechol (Table VII). This enzyme differs from laccase in that it does not catalyze the oxidation of hydroquinone, vanillin and p-phenylenediamine. In terms of substrate specificity, the phenolase complex of P.somniferum is identical to that of Keilin and Mann (74), Ludwig and Nelson (76) and Perkinson and Nelson (83) except that it does not oxidise tyrosine either in absence or in presence of added o-diphenol such as catechol. It also differs from catechol oxidase of tea leaves (110) and tobacco leaves (111) which do not oxidise monohydric phenols like p-cresol and tyramine. Since the phenolase complex of P.somniferum oxidises p-cresol without added o-diphenols, it differs from the enzyme of Bordner and Nelson (112). The substrate specificity studies suggest the presence of a distinct phenolas complex in P.somniferum extracts. Manometric studies and the studies on the formation of the products have shown that plant extracts are free from laccase and amine oxidase. The Phenola:

complex is widely distributed in all the tissues of P.somniferum. Very high concentrations of this enzyme in buds and fruits suggest that this enzyme might be involved in protective coating and functional as well as adventitious browning (96).

The optimum pH for monophenol oxidation has been found to be 7.8. with crude as well as partially purified enzyme preparations. This is in agreement with the results obtained by James et.al. (102) with belladona enzyme. With acetone powder extract, the pH-activity curve of DOPAMINE showed two optimum values at 6.2 and 8.6. This might be due to the association of non-specific protein with the enzyme molecule which might be dissociated at different hydrogen ion concentrations thus facilitating the enzyme substrate complex formation Fig. 14).

The Phenolase complex of P.somniferum is inhibited by semicarbazide, hydroxylamine and sodium sulphite. The inhibition observed by heavy metal reagents such as sodium azide and potassium cyanide suggested that the enzyme is a metallo-protein. The inhibitory effect of sodium diethyldithiocarbamate

salicylaldoxime, potassium ethyl xanthate and thiourea suggested that Phenolase complex of P.somniferum may be a copper containing protein. The strong inhibitory effect of thiourea suggested and confirmed the absence of plant amine oxidase in P.somniferum since thiourea is known to activate plant amine oxidase (97). That phenolase complex of P.somniferum is a copper containing protein was further supported by observations that the activity was inhibited by Ag^{+}, Au^{+++}, and Hg^{++} ions which are known to compete with copper (113).

The complete recovery of activity of sodium diethyldithiocarbamate inhibited enzyme by added cupric ions fully supported the view that phenolase complex of P.somniferum is a metalloprotein containing copper. Tenebaum and Jensen (100) suggested that copper is presumably bound to the enzyme protein by coordinate bonds. The copper part of the molecule is probably the chief anchoring group. The fixing of the free bonds of copper part of enzyme by addition of certain reagents leads to inactivation. The liberation of these bonds by cupric ions leads to reactivation. It is unlikely that any loss in activity and its

reversal are due to first reduction and then oxidation of cupric part of the molecule.

The inhibition of the enzyme by Hg^{++} might be due to its competition with Cu^{++}. The progressive recovery of the activity by added glutathione and cysteine may be due to the gradual removal of mercury by binding with it.

The inhibition of partially purified preparations of enzyme by potassium cyanide and 8-hydroxyquinoline suggested that this enzyme might have a role in terminal oxidation (114).

The enzyme was inactivated during the course of reaction. These observations are in accordance with those observed by many workers using different sources of enzyme (76,77,79,93,101,102). According to Ludwig and Nelson (76) the inactivation is not due to product inhibition but occurs as soon as catechol is oxidised. According to Nelson and Dawson (78) this is due to some factor inherent in catechol- enzyme- oxygen system.

The oxidative inactivation of P.somniferum phenolase complex itself during the oxidation of its substrate has been demonstrated. This is a well documented but little understood

phenomenon (115,116). Phenolase complex not only itself is inactivated during its action but can inactivate other enzymes as well. Schroeder and Adams (117) and Jonnard and Thompson (118) have showed the partial inactivation of renin by this enzyme in absence of added phenolic compound. More accelerated inactivation of renin by phenolase complex in presence of DOPA or catechol has been demonstrated by Schroeder and Adams (117) and Soloway and Oster (119). Inhibition of pepsin, trypsin and chymotrypsin in presence of phenolase complex-catechol system has been observed by Sizer and Brindley (104). Kertsz and Caselli (120) have shown that lysozyme is completely inactivated by phenolase complex catechol system even when separated from the oxidase by a cellophane membrane.

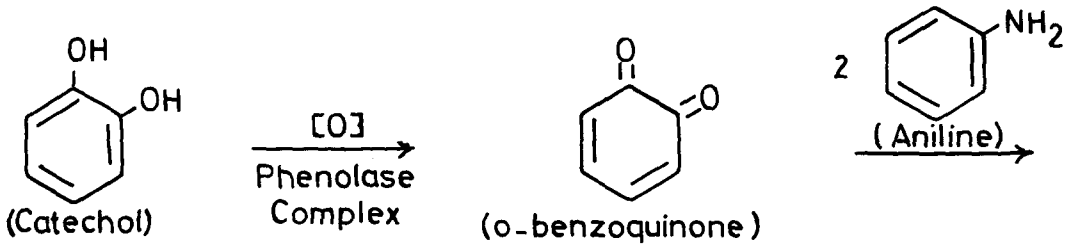
Sizer (121) believed that this inactivation might be due to the attack of this enzyme on the phenolic hydroxyl group available at the surface of the protein molecule. His studies have shown that only a small fraction of the total tyrosine of the protein is oxidised by tyrosinase and that each tyrosyl group does not appear to undergo a very extensive oxidation.

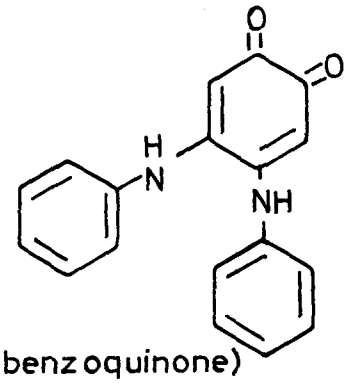
This suggests that other groups on the protein molecule such as amino, imino, sulfhydryl and free heterocyclic groupings might be involved (122).

The inhibition of ascorbic acid oxidase by phenolase complex of P.somniferum is an interesting finding in the present context. In view of the fact that oxidative enzyme like phenolase complex has been shown to act on certain proteins in vitro, it seems possible that other nonproteolytic enzymes may also attack native proteins. In particular the action on proteins of such enzymes as deaminases, decarboxylases, dehydrogenases and certain oxidases deserve careful study. Such investigations may provide a partial interpretation of the great liability of proteins in living system.

Manometric and iodometric studies have shown that o-quinones are formed by the action of P.somniferum extracts on various phenols. It was therefore desirable to isolate o-quinones from reaction mixture. The anilinoquinones produced by the action of phenolase complex on catechol in presence of aniline have been isolated and identified. The reactions

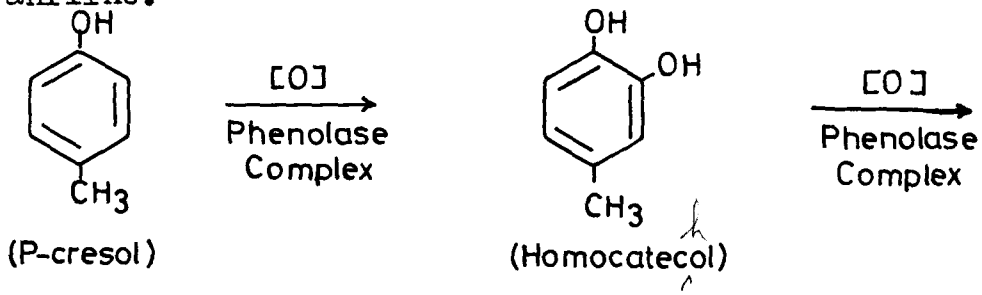
leading to the formation of dianilno- o-benzoquinone may be represented as follows:

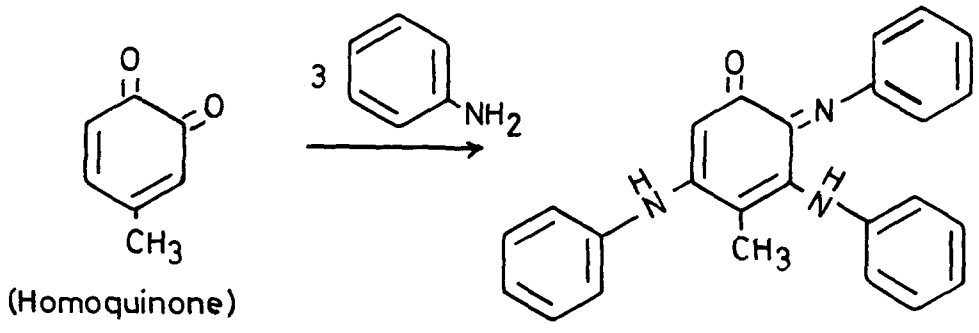




(Dianilino-o-benzoquinone)

The dianilinohomoquinoneanil could be obtained by the oxidation of p-cresol in presence of phenolase complex and aniline.

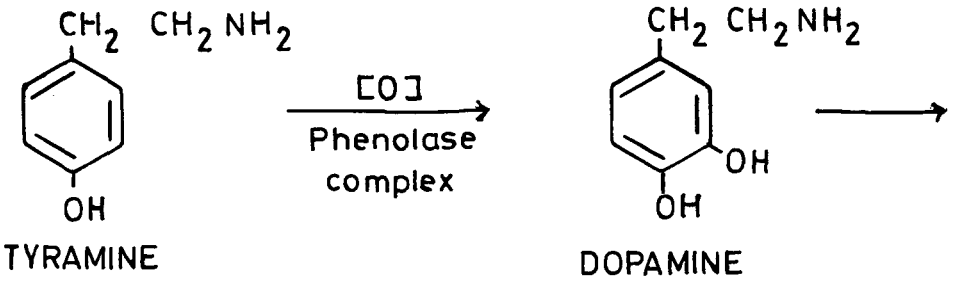


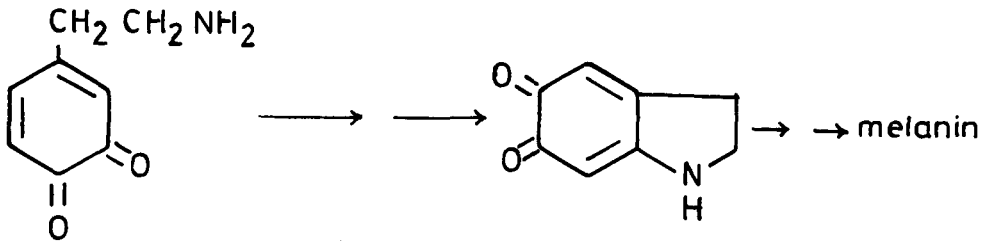


(Dianilinohomoquinoneanil)

It appears that the first stage in the action of phenolase complex on the monohydric phenols is the introduction of a second hydroxyl group in the ortho- position to the first, producing a catechol derivative which is then oxidized to corresponding quinone.

The oxidation of tyramine and DOPAMINE, the formation of quinones and eventual deposition of black reaction products suggested that the following pathway might be operative in opium plant.





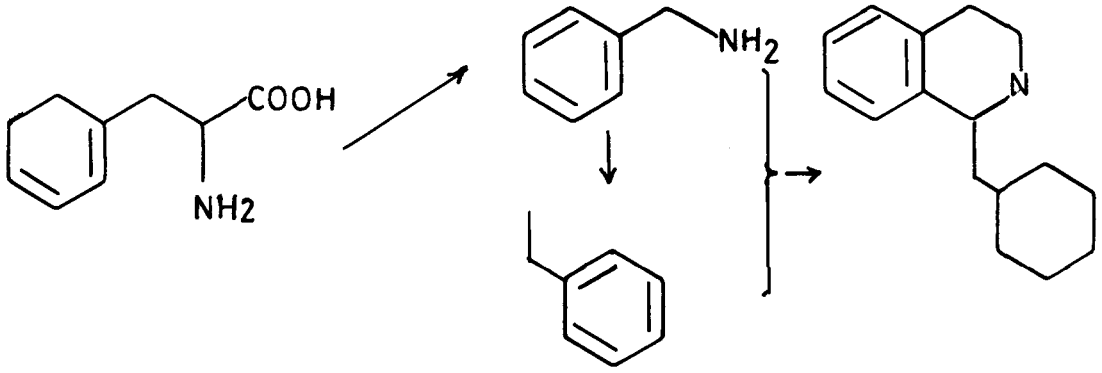
The oxidation of DOPA by P.somniferum acetone powder extract was accompanied with carbon dioxide evolution and decarboxylation was completely inhibited under nitrogen atmosphere (Fig. 8). This led us to believe that some oxidation product of DOPA is decarboxylated. Therefore the oxidation of DOPA and its transformationto melanin may be envisaged as shown in Fig. 1.

Analysis of the Km values with partially purified preparations showed that the phenolase complex has higher affinity for DOPAMINE and DOPA in comparison to other mono- and dihydroxy-phenols.

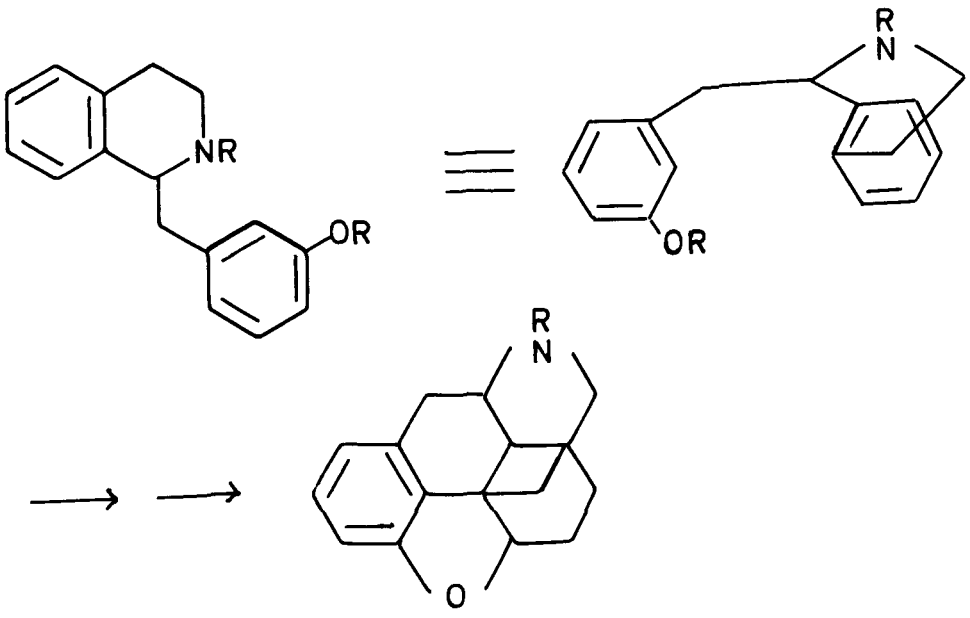
It seems appropriate at this time to comment on the relationship of the present work to the biogenesis of opium alkaloids. Most of the theories on the formation of morphine type alkaloids are variations of a scheme derived from suggestions of Winterstein and Trier (123) who were the first to suggest that phenylalanine could serve as the precursor of phenylethylamine and phenylacetaldehyde derivatives which could

combine to give the skeleton of the benzylisoquinoline alkaloids (eqn. VII)

Equation VII:



Equation VIII:



Gulland and Robinson (124) proposed intramolecular aromatic

coupling reaction of the benzylisoquinoline to furnish the hydrophenantherine skeleton (Eqn. VIII). These and subsequent theories (125,126,127,128,129,130) included attempts to establish more detailed mechamisms.

Battersoby and Harper (131) and Leete (132) independently fed C14- labelled tyrosine to P.somniferum and observed the formation of radioactive morphine, a hydrophenantherine alkaloid. Battersby and Bink (133) showed that specifically labelled norlaudanosine when fed to mature P.somniferum, radioactive morphine could be isolated from the plants. This led to the belief that tyrosine is the precursor of morphine. But this could only be said with certainity if norlaudanosine could be established definitely as one of the natural biosynthetic intermediates. Stermitz and Rapoport (134) were the first to point out that norlaudanosine might be a substance completely foreign to P.somniferum, as it has not yet been isolated as natural product. Further argument advanced by Stermitz and Rapoport against the basic biogenetic schemes (Eqn. VII and VIII) was that feeding labelled tyrosine to plants

gave very low yields of radioactive morphine.

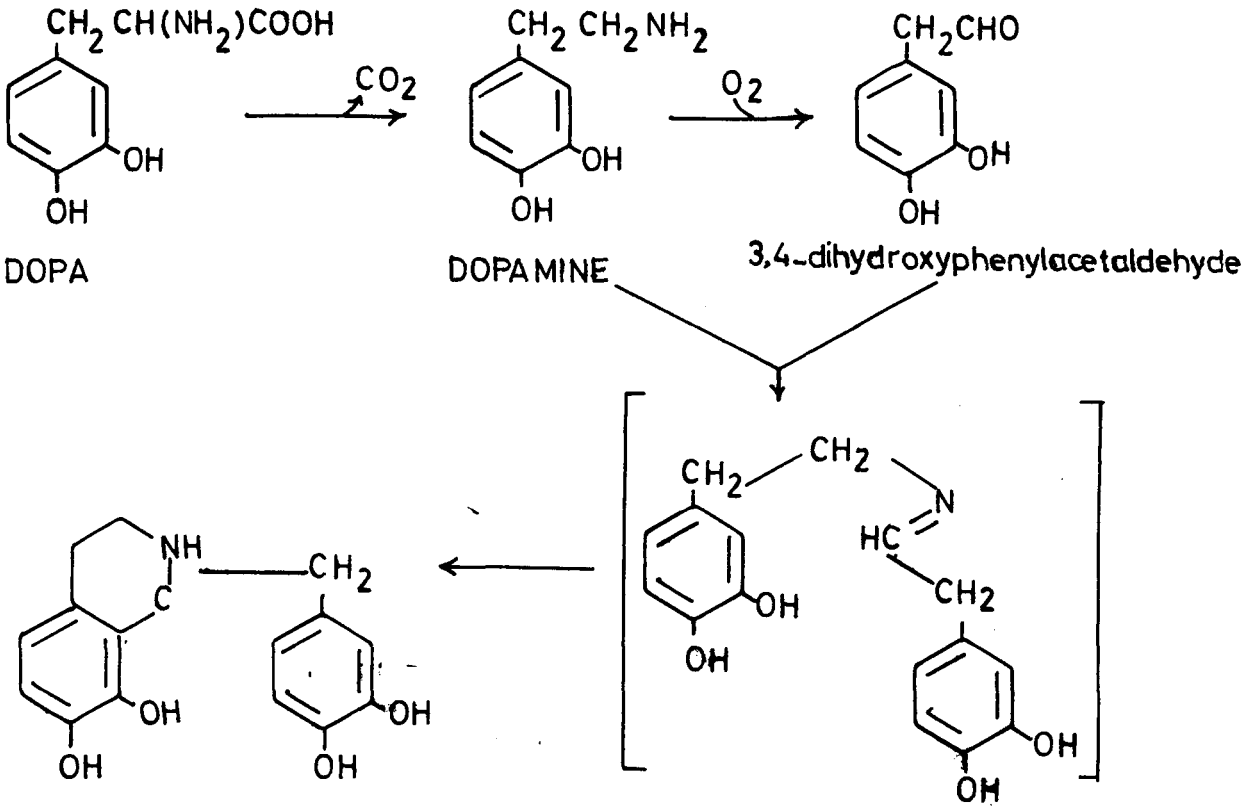
If tyrosine is a major precursor of opium alkaloids it would be expected to give better incorporation and yeilds than reported. In presence of $C^{14}O_2$ the incorporation as well as yields of morphine was much higher than when tyrosine was fed to P.somniferum (135). The incorporation achieved was more in line with what might be expected from a minor precursor or even through an aberrant synthesis. This possibility is also raised by the interesting results of Kleinschmidt and Mothes (136) who showed a higher incorporation into alkaloids of P.somniferum from glucose than from tyrosine, although generally accepted metabolic pathways indicate that tyrosine is formed from glucose. On the basis of these studies, it is felt that new proposals are required for biosynthisis of benzylisoquinoline as well as phenantherine type of alkaloids.

Another confusing aspect of the problem is that tyrosine-2-C^{14} as well as phenylalanine-2-C^{14} give rise to morphine group of alkaloids in P.somniferum (137,138). These results appear to be somewhat baffling in that there seems to be no

reasonto believe that tyrosine is formed from direct hydroxylation of phenylalanine in plants. Both amino acids appear to originate from independent pathways in plants (139). This suggests that incorporation of both amino acids in nor-laudanosine as well as morphine group of alkaloids is perhaps through an aberrant synthesis.

It seems therefore that at present two contrasting views are existing about the biosynthesis of nor-laudanosine of morphine. First, that these alkaloids are derived from tyrosine or their biochemical equivalent and second, that these alkaloids are not derived from tyrosine or its biochemical equivalent. To confirm any of these possibilities it becomes necessary to study these biogenetic sequences purely from enzymological point of view. The formation of nor-laudanosine, according to the detailed scheme of Robinson must consist the

following sequential steps.



The first two steps of this scheme must be catalyzed by DOPA decarboxylase and the amine oxidase respectively if it is operative in P.somniferum plant. The absence of DOPA decarboxylase as well as amine oxidase suggested that perhaps Robinson's scheme for the biogenesis of laudanosine is not operative in opium plant and supported Rapoport's suggestions that reported incorporation of tyrosine and phenylalanine into opium alkaloids is either through minor pathways or due to aberrant synthesis.

VIII. S U M M A R Y

1. The crude homogenates of roots, leaves, stems, fruits, buds and whole seedlings of P.somniferum or acetone powder extracts of whole plant are capable of oxidising DOPA. The oxidation of DOPA by the plant extracts suggested the presence of amino acid oxidase, amine oxidase or phenolase complex in P.somniferum. But since tyrosine and phenylalanine were not oxidised over a wide range of pH (5.0 to 9.0) it was unlikely that the oxidation of DOPA is due to amino acid oxidase.

2. Rapid evolution of carbon dioxide by the crude homogenates of roots, leaves, stems, fruits and whole seedling extracts of plant in presence of DOPA has been observed. This suggested the possibility of the presence of DOPA decarboxylase in the plant. But since this decarboxylation was accompanied with oxidation, it was also possible that first DOPA is oxidised and the resulting product is then decarboxylated. The complete inhibition of decarboxylation of DOPA under nitrogen atmosphere suggested that carbon dioxide evolution may not be due to decarboxylation of DOPA itself but it could be due to decarboxylation of some oxidation product of DOPA.

3. To test the possibility that oxidation of DOPA might be due to the action of amine oxidase, a search for the presence of amine oxidase in P.somniferum was made by studying the oxidation of various amines by the extracts. None of the

amines except those which contained hydroxyl group in the aromatic nucleus (DOPAMINE and tyramine) were oxidised. Cadeverine and putrescene, the best substrates of amine oxidase were not at all oxidised. The failure to demonstrate the presence of amine oxidase in P.somniferum suggested that the observed oxidation of DOPA was perhaps due to phenolase complex.

4. The oxidation of DOPAMINE and tyramine was not accompanied with ammonia and hydrogen peroxide formation. Thus the oxidation of these substrates could not be due to amine oxidase activity.

5. To test the possibility that the oxidation of DOPA, DOPAMINE and tyramine by the extracts of P.somniferum might be due to phenolase complex, the ability of the extracts to oxidise other phenolic compounds was studied. The crude homogenate of the plant oxidised catechol and p-cresol. However, m-cresol was not oxidised. Tyrosine was not oxidised even in presence of added catechol. These results suggested the presence of a distinct phenolase complex in P.somniferum which is different from the tyrosinases so far known. It is also different from laccase because it does not oxidise hydroquinone, vanillin and p-phenylenediamine.

6. The oxidation of DOPAMINE was strongly inhibited by thiourea. The inhibitory effect of thiourea suggested the absence of

plant amine oxidase in P.somniferum since thiourea is known to activate the amine oxidase.

7. The phenolase complex of P.somniferum is inhibited by semicarbazide, hydroxylamine and sodium sulphite. The inhibition observed by sodium azide and potassium cyanide suggested that the enzyme is a metalloprotein. The inhibitory effect of sodiumdiethyldithiocarbamate, salicylaldoxime, potassium ethylxanthate and thiourea showed that phenolase complex of P.somniferum may be a copper containing protein. The phenolase complex of P.somniferum is a copper containing protein was further supported by observations that the activity was inhibited by heavy metal ions like Ag^{+}, Hg^{++} and Au^{+++} which are known to compete with copper. These results together with other observed evidences suggest that phenolase complex of P.somniferum is a copper containing protein.

8. The enzyme was inactivated during the course of reaction. These observations are in accordance with the work of earlier workers. The mechanism of inactivation is not clearly understood.

9. Phenolase complex not only itself is inactivated during the course of its action but can inactivate ascorbic acid oxidase as well. Although the reason for such inactivation

is not known but further investigations may provide a partial interpretation of the great lability of proteins in living system.

10. The formation of quinones during the oxidation of DOPAMINE, DOPA, catechol, p-cresol and tyramine by extracts of P.somniferum has been demonstrated. Dianilino-o-benzoquinone and dianilinohomoquinoneanil produced by the action of phenolase complex on catechol and p-cresol, respectively, have been isolated and identified. It is believed, therefore, that first step in the oxidation of monophenols is o-hydroxylation resulting in the formation of diphenols and o-benzoquinones are formed by the oxidation of diphenols.

11. The oxidation of DOPAMINE, DOPA and tyramine, formation of o-benzoquinones and eventual deposition of black reaction product suggested that the pathway of melanin biosynthesis may be operative in P.somniferum.

12. The phenolase complex has been purified 47-fold from the acetone powder extracts of P.somniferum using ammonium sulphate precipitation method, CM-cellulose and DEAE-cellulose chromatography. The properties of purified preparations have been studied. The substrates, DOPAMINE, DOPA, catecho, p-cresol and tyramine which were oxidised by crude homogenates were also oxidised by partially purified preparations. The pH-activity curves of monophenols e.g., p-cresol

and tyramine show optimum activity around pH 7.8. The diphenols on the other hand show lack of well defined optimum pH with maximum oxygen uptake around pH 7.5 (DOPAMINE and DOPA) and pH 6.0 (catechol). Analysis of the Km values showed that phenolase complex of P.somniferum has highest affinity for DOPAMINE. Higher concentrations of tyramine were inhibitory for the enzyme activity. The enzyme was inhibited by copper chelating agents. It was also inhibited by respiratory inhibitors like cyanide and 8-hydroxyquinoline.

13. The presence of phenolase complex in relation to melanin biosynthesis and the failure to demonstrate the presence of DOPA decarboxylase and amine oxidase in P.somniferum in relation to the biogenesis of opium alkaloids have been discussed.

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